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THE FERMENTATIVE PRODUCTION OF GLYCEROL

by

Thomas M. Lees

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Biophysical Chemistry

Approved:

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I. INTRODUCTION

Glycerol is one of the most important industrial chemicals. Indicative of the importance of this compound, some statistics are shown in Tables I and II concerning the imports, exports, and production of glycerol. It is interesting to note the sharp drop in imports and the corresponding increase in exports during the two war periods. The need for glycerol during the present crisis is clearly evidenced by the current fat salvage campaign.

Table I

Glycerol Production in the United States*

Year	Refined glycerol (lbs.)	Net cost per lb.
1929	113,140,075	$ 0.11
1931	102,509,832	0.10
1933	107,852,796	0.07
1935	118,726,871	0.11
1937	122,399,454	0.17
1939	158,778,320	0.104

*Manufacturing Chemists' Association (1940)

According to Lawrie (1928) the production of 80 per cent crude glycerol in the United States rose during the years from 1919 to 1925 from 61,792,958 pounds to 103,406,943 pounds. This increase reflected the development of new uses

for glycerol.

Table II

Imports and Exports of Glycerol

Year	Imports		Exports
	Crude (lbs.)	Refined (lbs.)	(lbs.)
1913*	34,399,129	432,339	---
1914	36,230,383	551,306	---
1915	18,661,929	360,567	---
1916	10,875,058	303,463	---
1917	4,078,803	137,128	---
1918	1,925,815	19,750	21,045,991
1919	1,163,952	162,916	13,018,882
1920	15,664,109	1,911,278	2,257,623
1936**	11,148,985	3,447,487	1,146,026
1937	13,441,430	7,535,120	1,375,036
1938	13,097,525	2,567,411	3,746,217
1939	10,987,731	330,078	7,398,681

*Lawrie (1928)

**Manufacturing Chemists' Association (1940)

Some of the important uses of this versatile chemical are in the production of high explosives, anti-freeze mixtures, medicinals, plastics, and as a tobacco-moistening agent. Lawrie (1928) lists ninety-two products in the manufacture of which glycerol is used, and this list is not a complete one. Large amounts of glycerol are also used in the manufacture of ester gums for varnishes, as a plasticizer in cellophane, and as a lubricant for certain machines in the food-processing industries.

From the average statistics for the period 1940-41 the

major uses of glycerol were as follows (adapted from U.S.I. Chemical News [1943]):

Nitroglycerine	18.8%	Cellophane	14.2%
Resins	16.0%	Edible	9.7%
Tobacco	14.6%	Drugs	6.8%

All of the glycerol available in the past has been obtained by the saponification or hydrolysis of fats and oils. In recent years synthetic methods for the production of glycerol have been worked out, for example the one by Williams (1940), but no data are available to show to what extent synthetic processes are being used, if at all. The amount of glycerol produced by the saponification or hydrolysis of fats and oils is, of course, limited by the supply of fats and oils and is also dependent on the market for soap and candles. The glycerol synthesized by Williams' process requires propylene, a material derived from the propane in natural gases which are not inexhaustible and represent a capital asset in our natural resources. Another method for the preparation of glycerol involves the alteration of the course of a normal alcoholic fermentation by the use of sulfites, alkalies, or even neutral salts. Fermentation methods have the advantage that the fermentable substrate is derived from agricultural products which represent an annual income of matter and energy.

At the beginning of the last war the Germans, realizing the need for glycerol in making high explosives, began to

develop fermentation methods for the production of glycerol based on the theoretical investigations of Neuberg (1913). Connstein and Lüdecke (1921) undertook the technical production of glycerol on a large scale, and Lawrie (1928) devotes considerable space to describing the process both as to fermentation and recovery. According to this description, the Protol Gesellschaft was organized and about twenty-four factories set about making glycerol by fermenting pure white beet sugar in the presence of sodium sulfite. The smaller factories shipped the fermented slop to the larger ones for the recovery of the glycerol. About 10 to 12 kg. of best refined sugar were required in the production of one kilogram of dynamite glycerol. No commercial scale production of fermentation glycerol was carried on in the United States during the last war, although Eoff (1919) conducted some pilot plant fermentations using sodium carbonate instead of sodium sulfite. With the cessation of hostilities the German sulfite process was probably discontinued because of the relatively higher cost of the fermentation product and the shrunken market. The higher cost of the fermentation product could be attributed to the cost of the substrate and to the high losses suffered in recovering the glycerol. The fact that the glycerol produced by the soap manufacturers can be considered as a by-product and the price adjusted accordingly would also tend to keep the fermentation product off the market. However, in times of emergency such as the last war

and even in the present one fermentation methods do offer a solution to the shortage of glycerol. Degering (1943) mentions that a modification of the sulfite process has been developed by the Tennessee Eastman Corporation for the commercial production of glycerol, but does not say whether it is actually being used.

Since about 1922 few important developments have taken place in the methods of producing glycerol by fermentation. A good deal of work has centered around the problem of recovering the glycerol once it is formed. Some of the newer developments in distilling and extracting glycerol are much more efficient than the older methods of recovery. The two hindering factors in the recovery of the glycerol are the high concentrations of salts present in the fermented beers and the presence of gums, dextrins, unfermentable sugars, and so forth, carried over from the original sugar source, usually cane or beet sugar molasses.

Hickey (1941) described a fermentation procedure for glycerol production employing slightly soluble sulfites which could be removed after fermentation. In this process magnesium or calcium sulfite is used in place of the more soluble sodium sulfite. The pH is adjusted to the proper level and the fermentation is carried out as usual with the difference that agitation is necessary to keep the solid sulfite suspended and in contact with the medium. Yields of glycerol are higher when the more soluble magnesium salt is used, but

in neither case do the yields go as high as those in the sodium sulfite fermentations. The calcium and magnesium salts are removed by filtration with subsequent addition of magnesia or lime to decompose the aldehyde-bisulfite complex liberating the acetaldehyde and precipitating any remaining calcium or magnesium ions remaining in solution. Sodium ions cannot be removed by such procedures because of the general solubility of most sodium salts. Starting with a reasonably pure sugar the beer remaining after a calcium or magnesium sulfite fermentation should offer fewer problems in recovery.

The use of a pure sugar as a substrate in the glycerol fermentations would simplify recovery but it would also add to the initial cost. No reports were found in the literature on the use of solutions of acid-hydrolyzed starch or grain as sources of sugar to be fermented directly without recovering the dextrose from solution, with the exception of a brief comment by Eoff (1919) on the use of corn syrup. It is interesting to note that several patents claim the use of maltose as a fermentable sugar in the glycerol fermentation, but no reports of experiments were found on the use of maltose or of a malted grain mash as the sugar source. For instance, Connstein and Lüdecke (1921) claim the use of a saccharified starch mash in their patent but no examples were given of its use. In a private communication R. J. Hickey (1943) emphasized the fact that such mashes gave poor fermentations and needed special handling.

In the patent literature, and to some extent in the journal reports, yields of glycerol up to the theoretical 51 per cent have been claimed. Yet reports on large-scale fermentations seem to indicate that yields of 20 to 25 per cent glycerol on hexose would be considered good. Of about thirty yields above 15 per cent reported in different articles and patents, two-thirds were in the range from 15 to 28 per cent, the remainder ranging from 30 to 42 per cent. There are several possible explanations for these varied results. The methods of analysis may have been different; recovery methods especially are susceptible to large errors. It was noted that in most cases high yields were associated with abnormally large inocula. It is common practice in fermentation processes to use a fermented beer as an inoculum, but in many cases laboratory workers have used pressed yeast obtained from a convenient brewery or distillery as an inoculum when investigating the glycerol fermentation.

The purpose of the work on which this thesis is based was to investigate the possibility of using starchy materials as the source of fermentable sugars for the glycerol fermentation and to investigate further the various factors which influence the yield of glycerol. During the investigation the limits of practical application of the fermentation process were kept in mind. Such limits would include the time of fermentation, ease of recovery, size of inoculum, and availability of the fermentable substrate.

II. REVIEW OF PREVIOUS INVESTIGATIONS

A. General Discussion

The literature pertaining to glycerol has been rather completely covered, up to the time of printing, by Lawrie (1928) in his excellent monograph. The material for the following abbreviated discussion of the discovery and early history of glycerol was obtained from Lawrie's monograph.

Glycerol, more commonly called glycerine, is a viscous, colorless and almost odorless liquid which is found free in nature only to a very limited extent. Some free glycerol is found in palm oil and to an even lesser degree in some rarer oils. Most glycerol is found in fats and oils in combined form and is obtained from them by saponification or hydrolysis. The honor of the discovery of glycerol belongs to Scheele, who in 1779 first prepared this compound by mixing olive oil and litharge and then heating the mixture. Upon washing with water, a sweet-tasting solution was obtained which, on evaporation of the water, gave a viscous liquid. He called this new substance "the sweet principle of fats." It was known also as Scheele's sweet principle or as oil sugar. Chevreul in 1811 studied the composition of this sweet liquid and named it glycerol. For many years Scheele's process was the only commercial method for producing this compound, but as uses

for glycerol became more general, the demand increased and a new method was sought for its preparation. In 1823 Chevreul obtained a patent wherein alkalies were used to decompose the fats and thus a new method for the production of glycerol was developed. Later work by Tilgham showed that at high temperatures and under pressure the acid or alkali might be dispensed with. The second great development in the production of glycerol was the discovery by Wilson in 1856 that glycerol could be distilled with steam. In 1868, Nobel, following Sobrero's discovery of glyceryl trinitrate, found that nitroglycerine, as it is more commonly called, could be handled more safely after it had been absorbed by kieselguhr. Thus a great market was opened for glycerol in the production of dynamite and also blasting gelatine.

Glycerol contains a hydroxyl group on each of three adjacent carbon atoms, the formula being $CH_2OH \cdot CHOH \cdot CH_2OH$. As can be seen, there are two primary alcohol groups and one secondary alcohol group. As an alcohol it may react to produce esters and ethers. Advantage is taken of this reaction in the production of the glyptal resins. It resembles the trioses in that it has a sweet taste. Glycerol is a weak acid and dissolves the alkalies, the alkaline earths, and many metallic oxides, forming with them metallic compounds similar to the alcoholates, or addition products in the nature of double salts. At high temperatures glycerol tends to decompose to form acrolein and other decomposition products. This

tendency is noticeable at the boiling point, 270° C., and therefore distillations of glycerol are best conducted under vacuum and in the presence of steam. The presence of salts also accelerates the decomposition of glycerol. Glycerol is infinitely soluble in water and alcohol, but insoluble in ether and chloroform. Extraction procedures for recovering glycerol are complicated by the fact that most of the solvents for glycerol will also dissolve water, and therefore sugars and salts tend to be carried over. Various reports are to be found on the use of butyl alcohol and even ethyl alcohol in extraction procedures to recover the glycerol. Advantage is taken of the high hygroscopicity of glycerol in the manufacture of certain inks and as a moistening agent for tobacco.

Practically all of the glycerol produced at present is obtained by the decomposition of fats and oils. All fats and vegetable oils are glycerides of organic acids usually occurring as mixtures. The saponification or hydrolysis of these compounds may be brought about in various ways. The most important of these methods which are in commercial operation are:

1. Saponification by caustic soda;
2. Saponification or hydrolysis by steam under pressure with or without the addition of catalysts;
3. Saponification at ordinary pressures in the presence of a special catalyst;
4. Enzymatic splitting to glycerol and fatty acids.

The bulk of the world's supply is obtained from the soap-making process in which caustic soda is used.

Wurtz (1857) was one of the first to synthesize glycerol. In his method 1,2,3-tribromopropane was treated with silver acetate to give triacetin which on hydrolysis gave glycerol. There are numerous other synthetic methods most of which utilize propylene as the initial starting material. The process developed by Williams and his coworkers (1938, 1940, 1941) is probably the best and is reported to have been run on a small scale at a profit. In this process propylene is chlorinated under high pressure to form allyl chloride which in turn is converted to allyl alcohol by means of sodium hydroxide. Hypochlorous acid is added to the unsaturated linkage to form the chlorohydrin which is then hydrolyzed to glycerol. In view of the present availability of appreciable amounts of propylene in the gases from petroleum-cracking plants, together with the low cost of alkalies and chlorine, it might seem that the synthetic method would be quite feasible. However, the initial cost and the upkeep of a plant of this type would be high; the supply of propylene from the aforementioned sources is limited, and the supply of petroleum itself is not without end as we are now well aware. Another drawback to the method is that the glycerol produced by it is contaminated, even in its purest form, by small amounts of glycols and polyglycols which might be disadvantageous in the pharmaceutical and cosmetic industries. Levey (1938) and Williams (1941), both of whom are more in favor of synthetic methods than of fermentation procedures, have discussed the economics of the

production of synthetic glycerol.

A series of patents relating to the production of glycerol by the hydrogenolysis of carbohydrates has been granted to the Association of American Soap and Glycerine Producers, Incorporated (1937, 1939). In the hydrogenolysis process a polyatomic aliphatic alcohol such as sucrose is treated under pressures of about 145 atmospheres and at temperatures above 145° C. with hydrogen in the presence of a catalyst and a carrier. In the case cited a copper aluminate catalyst was described and anhydrous methanol was used as the carrier. Sucrose was converted to 45.8 per cent propylene glycol, 21.5 per cent glycerol, and 6.8 per cent of less volatile glycerol-like compounds. Such a mixture could be used as an anti-freeze. Hass and Patterson (1941) described a method of purifying glycerol obtained by hydrogenolysis through crystallization from butyl alcohol.

B. Preparation of Glycerol by Fermentation

1. The use of sulfites

Pasteur (1858) was the first to identify glycerol as one of the products formed when yeast acted upon sugars. In his investigations on the production of wines and beers Pasteur found that 3 to 5 gms. of glycerol were formed from 100 gms. of sugar. It was not until Neuberg and his associates (1913, 1917, 1919) began to publish their work on the

mechanism of the alcoholic fermentation that advantage was taken of this potential source of glycerol. The advent of the first World War also gave impetus to the studies. Nord (1926) has aptly said, "Here is displayed a not frequent case where practical workers have very fruitfully and stimulatingly influenced theoretical research in this sphere." Lawrie (1928) discussed the production of glycerol by fermentation quite thoroughly in Chapter V of his excellent monograph, and recently Whalley (1942) has put out a compilation of abstracts on this subject. Shorter reviews of the glycerol fermentation are to be found in the literature by May and Herrick (1930), Prescott and Dunn (1940), and Owen, Levey and Owen (1940).

Based on his own research and that of his associates (1913, 1917, 1919, 1922), Neuberg conceived the yeast fermentation of glucose as being capable of taking place in three different ways depending on the conditions of the fermentation. According to his views, all three of these forms involved the breakdown of the hexose into two molecules of hydrated methylglyoxal which was then transformed to pyruvic acid, setting free active hydrogen according to the equation,

$$C_6H_{12}O_6 \rightarrow [2]CH_3CO\cdot CH(OH)_2 \rightarrow 2CH_3CO\cdot COOH + [2]H_2$$

The pyruvic acid was then attacked by the enzyme carboxylase and broken down into acetaldehyde and carbon dioxide, as follows:

$$CH_3CO\cdot COOH \rightarrow CH_3CHO + CO_2.$$

In a normal alcoholic fermentation the acetaldehyde was finally reduced by the active hydrogen to give ethyl alcohol, the overall equation being

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad \text{(Neuberg's first form).}$$

However, if an aldehyde fixing agent was added to the fermentation medium, the acetaldehyde could not be reduced by the hydrogen which therefore reduced the methylglyoxal hydrate to form glycerol according to the equation

$$CH_3CO{\cdot}CH(OH)_2 + H_2 \rightarrow CH_2OH{\cdot}CHOH{\cdot}CH_2OH \quad \text{(glycerol)},$$

the overall equation then becoming

$$C_6H_{12}O_6 \rightarrow C_3H_8O_3 + CH_3CHO + CO_2 \quad \text{(Neuberg's second form).}$$

In Table III some of the proof of this relationship is given.

Table III.

Yields of Glycerol and Aldehyde with Varying Proportions of Sodium Sulfite*

Used		Produced		Ratio of aldehyde to glycerol
Na_2SO_3 (parts)	Sugar (parts)	Aldehyde (parts)	Glycerol (parts)	
33	100	11.90	23.37	1:1.96
50	100	12.52	24.86	1:1.98
75	100	13.89	27.62	1:1.98
150	100	18.65	36.90	1:1.98

*Adapted from Lawrie (1928)

If the fermentation were carried out under alkaline conditions,

the acetaldehyde underwent a Canizzaro reaction to give acetic acid and ethyl alcohol. Since the hydrogen was again free to reduce the methylglyoxal hydrate, glycerol was again formed, this reaction, Neuberg's third form of fermentation, being formulated as follows:

$$2C_6H_{12}O_6 + H_2O \rightarrow 2C_3H_8O_3 + C_2H_5OH + CH_3COOH + 2CO_2.$$

Although Neuberg's final equations have seldom been disputed, his assumption of the existence of methylglyoxal was hypothetical, and in newer theories of fermentation it has been displaced.

The best explanation of the formation of glycerol by yeast is probably to be found in the Embden-Meyerhof-Parnas scheme of carbohydrate dissimilation. Essentially in this scheme the glucose is first phosphorylated to a hexosediphosphate which in turn is broken down into two trioses, dihydroxyacetone phosphate and glyceraldehyde phosphate, which are in equilibrium with one another. Under ordinary conditions the dihydroxyacetone phosphate is further transformed through several steps into pyruvic acid and then into a number of products, chief of which is acetaldehyde in the alcoholic fermentation. The acetaldehyde is further reduced to ethyl alcohol. In the case of the sulfite fermentation the acetaldehyde is bound in the form of the bisulfite addition product which is not reducible, and then the hydrogen which would otherwise reduce the aldehyde reduces the

glyceraldehyde phosphate to ∝-glycerophosphate, which in turn is dephosphorylated to glycerol. In the initial phases of the normal alcoholic fermentation, before the acetaldehyde is formed and acts as the prime hydrogen acceptor, the glyceraldehyde phosphate is reduced to glycerol. This latter process accounts for the 2 or 3 per cent of glycerol normally found in the alcoholic fermentation. Werkman (1942) and Nord (1926, 1940) discuss the above fermentation scheme at greater length in their excellent reviews.

Dumas (1874) earlier had shown that the alcoholic fermentation can proceed in the presence of various alkali salts and hinted at the possibility of influencing the course of the fermentation by the addition of alkali sulfites. Müller-Thurgau and Osterwalder (1914) observed that in the case of fermenting sugar solutions, added sulfurous acid instantly combined with something which they correctly assumed to be acetaldehyde. It was obvious that this compound might be the acetaldehyde-sulfurous acid complex found by Ripper (1892), the sodium salt which had been known since the time of Bunte (1873). Kerp (1904) found the dissociation constant of the acetaldehyde-sodium bisulfite complex to be 2.84×10^{-6} and that of the glucose compound to be 3.11×10^{-1}, a ratio of about 1:90,000 showing that the acetaldehyde compound is much more stable than the corresponding glucose compound.

Connstein and Lüdecke (1919, 1921, 1924), proceeding on the knowledge then available and stimulated by the contemporary

work of Neuberg, began to investigate the possibilities of the commercial preparation of glycerol by fermentation. At first their investigations, probably started about 1914 or earlier, centered about the use of alkaline salts such as di-sodium phosphate, ammonium carbonate, sodium bicarbonate, and so forth, but early in the work a disagreeable fact arose, namely that under these alkaline conditions other bacteria would grow quite well and therefore the mashes were often badly contaminated. With sodium sulfite this trouble was not encountered, since this compound exerts an antiseptic effect which yeast seem to tolerate to a higher degree than other organisms. Rahn and Conn (1944) claimed that the toxic factor to yeast was the undissociated sulfurous acid, 7 to 8 mgs. of the latter bringing about the rapid death of the yeast at a pH of 2.6 - 2.9. However, the concentrations used by the latter investigators were much lower than those ordinarily used in the sulfite fermentation, and the pH level too low. Connstein and Lüdecke found that the yield of glycerol increased proportionally to the amount of sodium sulfite used, up to a certain limit above which the fermentation proceeded too slowly and the yeast was harmed. Furthermore, they found that the yeast which does not grow or has but little growth could be reused if a non-sulfite fermentation was interposed between sulfite fermentations. Two factors seemed to influence the yield of glycerol: a general salt action, since neutral salts increased the yield of glycerol, and the specific action

of the sodium sulfite. The Connstein and Lüdecke process was turned over to the German army authorities and the Protol Gesellschaft was organized and began to produce glycerol by the sulfite process. In 1921 a United States patent covering their process was issued to Connstein and Lüdecke. There are five main claims in the patent:

1. The process for manufacturing propantriol which consists in adding alkaline sulfites (until alkaline reaction) and yeast to sugar and then allowing the mixture to be fermented.

2. The process for manufacturing propantriol which consists in adding alkaline sulfites (until alkaline reaction) and yeast to sugar, causing the sugar to be fermented in presence of alkaline sulfites, separating the yeast and adding the separated yeast and alkaline-reacting substances to sugar, whereupon the process is repeated.

3. The process for manufacturing propantriol which consists in adding alkaline sulfites (until alkaline reaction), neutral salts of magnesium in a higher amount than necessary as yeast nutrients, and yeast to sugar and causing the sugar to be fermented.

4. The process for manufacturing propantriol which consists in adding alkaline sulfites (until alkaline reaction) and yeast to sugar, causing a part of the sugar to be fermented, adding new portions of sugar and causing the sugar to be fermented.

5. The process of producing glycerol which consists in fermenting a solution of fermentable sugar in an alkaline reacting medium.

According to Connstein and Lüdecke, neither the kind of sugar nor the variety of yeast influenced the fermentation. Refined and crude sugars, as well as molasses, could be used. The following tables taken from Lawrie (1928) summarize some

of the results obtained by Connstein and Lüdecke in their investigations:

Table IV

Yields of Glycerol with Alkaline Salts*.

Alkaline Salt	Salt (parts by weight)	Sugar	Glycerol produced (per cent)
Disodium phosphate	46	100	11.0
Disodium phosphate	70	100	15.6
Ammonium carbonate	10	100	13.4
Sodium acetate	30	100	9.5
Sodium bicarbonate	14	100	12.7
Control, no salts added	--	100	3.0

*Adapted from Lawrie (1928)

Table V

Glycerol Fermentation Yields with Increasing Concentrations of Sodium Sulfite*.

Sodium Sulfite (parts by weight)	Sugar (parts by weight)	Glycerol Yield (per cent on sugar)
40	100	23.1
67	100	24.8
80	100	27.3
100	100	30.1
120	100	33.0
150	100	34.6
200	100	36.7

*Adapted from Lawrie (1928)

Table VI

Production of Aldehyde, Alcohol and Carbon Dioxide with Increasing Sodium Sulfite Concentration*.

Sulfite added (gms.)	Aldehyde (per cent)	Alcohol (per cent)	Carbon dioxide (per cent)
25	2.42	39.96	37.5
40	5.6	---	---
50	5.8	35.8	35.8
67	7.6	---	---
80	9.9	---	---
100	10.0	29.4	29.4
120	15.0	---	---
150	17.6	---	---

*Adapted from Lawrie (1928)

The Connstein and Lüdecke process for the preparation of glycerol was also applied on a large scale by the Austro-Hungarian government during the last war. Zerner (1920) reported on the investigations conducted in connection with the process. In a typical example 100 gms. cane sugar, 40 gms. sodium sulfite, 10 gms. yeast and nutrient salts were diluted to 800 ml. and fermented for about eighty hours at 35° C. The yields were 12.9 per cent acetaldehyde, 26.18 per cent glycerol, 37.3 per cent carbon dioxide and 23.7 per cent ethyl alcohol. In no case could the yield of glycerol be increased above about 38 per cent. Zerner disagreed with Neuberg's view that the impossibility of converting all of the sugar to the theoretical amount of glycerol could be attributed to the dissociation of the aldehyde-bisulfite complex.

Zerner took the viewpoint that the reaction between the sodium bisulfite and acetaldehyde was not instantaneous and therefore competition for the aldehyde existed between the sodium bisulfite and the reducing enzyme of the yeast. Contrary to Neuberg, Zerner claimed also that the pyruvic acid-bisulfite compound was not fermentable and could not be the precursor of the acetaldehyde in the fermentation. He had no way of anticipating at that time the present idea that phosphopyruvic acid is involved in this transformation.

Cocking and Lilly (1922) in England attempted to develop a process whereby the yields of glycerol could be raised above those obtained by Connstein and Lüdecke to the theoretical values demanded by the second equation of Neuberg. Their investigations centered about the fact that the bisulfite ion and not the sulfite ion is the ion which combines with the acetaldehyde. In an ordinary sulfite fermentation the bisulfite ion is produced by the action on sodium sulfite of the carbon dioxide formed by the yeast from the sugar; naturally the fermentation has proceeded to a certain degree before much bisulfite is formed. However, bisulfites are too toxic to add directly to the fermentation mashes. Cocking and Lilly found that they could add a mixture of sodium bisulfite and sodium sulfite, in such proportions as to give a neutral reaction to litmus, to a mixture of sugar and yeast and thus obtain glycerol. They claimed that no special acclimatization was needed for the yeasts used and that most yeasts were

suitable. The sodium bisulfite could also be added as the fermentation proceeded as long as the reaction to litmus was kept neutral. The highest yield reported by them was 48.6 per cent of the sugar fermented converted to glycerol. The latter value was arrrived at by calculating back from the amount of sulfite combined with the aldehyde; by actual analysis of the mash for glycerol, 45 per cent was found. In either case these are among the highest yields to be found reported in the literature.

It is interesting to note that Connstein and Lüdecke and also Cocking and Lilly attacked the problem of the glycerol fermentation from the viewpoint of making commercial application of their findings, while Neuberg and his coworkers were primarily interested in proving their theories of the alcoholic fermentation.

Gehle (1922) confirmed Neuberg's work on the equivalence between glycerol and acetaldehyde. One point emphasized by Gehle was that the toxic effect of sodium sulfite increased rapidly once the ratio of one mole of sulfite to one mole of the sugar had been exceeded. This probably was fortuitous, since the addition product of dextrose and sodium bisulfite is highly dissociated in water solution. The highest yield of glycerol found by Gehle, himself, was 29.5 per cent on the dextrose; this and other values agree rather well with some of the values obtained in the work for this thesis. Gehle also reported finding an excess of glycerol over the amount

equivalent to the acetaldehyde fixed, and he attributed this to the metabolic functions of the yeast. This "protein-glycerol" varied as the metabolic rate of the yeast, that is, more was present in a rapid fermentation than in a slower one, and the amount was dependent on those factors which effect the well being of the yeast cells. In fermentations lasting from three to five days Gehle found about 5 per cent more glycerol than the acetaldehyde fixed could account for. Whether this glycerol arose from some unknown process or from the reduction of glyceraldehyde phosphate in the initial stages of the fermentation before acetaldehyde acts as the hydrogen acceptor, or whether it arose from the alkaline fermentation of sugar according to the third form of alcoholic fermentation, as Neuberg stated it, is still open to question.

The following is a summary of some of the conclusions reached by Gehle.

1. With increasing sulfite concentration an increasing alteration of the fermentation products occurs. The alteration nearly ceases at about 60 per cent sulfite on sugar, although complete fermentation takes place at 100 per cent. Explanation: As the concentration of the hydroxyl increases conditions arise which result in an asymmetric decomposition of the sugar molecule. As the alkalinity of weakly alkaline salts reaches a limit rather quickly with increasing concentration, the alteration produced by this alkalinity must also reach a limit.

2. The amount of glycerol produced in a fermentation by living yeast cells exceeds that calculated from the aldehyde equivalence by an amount comparable to that produced in a normal alcoholic fermentation.

3. Different yeast strains have little effect upon the qualitative and quantitative decomposition of the fermentable substrate. The only difference is that different yeasts show varying resistance to sulfite toxicity and therefore the "protein-glycerol" may vary (a measure of the vitality of the cells). The "protein-glycerol" is produced by the inner metabolism of the cell which is only indirectly related to the processes going on in the cell periphery. The production of this glycerol depends on collective factors which are favorable or unfavorable to the yeast.

4. The total material accounted for (aldehyde, glycerol, alcohol, and carbon dioxide) is 80 to 85 per cent; including acetic acid it is about 85 to 90 per cent.

5. Comparison of fermentation activity with increasing sulfite concentration by means of carbon dioxide development shows that in lower concentrations the yeast strives to a maximum by slow increments. In higher concentrations the picture is reversed (around 60 per cent sulfite) so that only by a sharp break can the transition into the later stages take place. The yeast seeks to control the toxicity of the sulfite in the higher concentrations by a regulation of the diffusion.

The investigations mentioned previously utilized sodium sulfite and bisulfite, but according to Neuberg (1919) all sulfites may be used. However, since an excess of sulfite (in solution) is necessary to prevent excessive dissociation of the acetaldehyde-bisulfite addition compound, the yields of glycerol obtained by using some of the more insoluble sulfites can never be as high as those obtained by using the more soluble sulfites such as sodium sulfite. Calcium and magnesium sulfite were used by Neuberg primarily. The former is less soluble than the latter. The use of the insoluble calcium sulfite enabled Neuberg to investigate the intermediary

metabolism of those organisms which are sensitive to alkaline media and to high concentrations of soluble salts. As the sulfite in solution is fixed by the acetaldehyde, more of the insoluble sulfite will dissolve to maintain equilibrium, and therefore there will never be at any one time an excessive amount of soluble sulfite present. The insoluble salt present acts as a reservoir. By using calcium sulfite and vigorously agitating the medium both mechanically and by passing carbon dioxide into the medium to keep the calcium sulfite in suspension, a yield of 14.2 per cent glycerol on hexose weight was obtained.

By conducting the fermentation in a medium acidified with phosphoric acid a yield of 15.2 per cent glycerol on hexose was obtained. This latter experiment proved an interesting point since previous fermentations using sodium sulfite had been conducted in media which were alkaline or neutral to litmus.

Hickey (1941) investigated the use of ammonium, magnesium, and calcium sulfites in the production of glycerol, paying particular attention to the pH of the fermenting media. Control of the pH had not been previously investigated to any great extent. Ammonium sulfite would have an advantage in the glycerol fermentation in that the ammonium and sulfite ions could be removed readily by volatilization and precipitation respectively, thus expediting the recovery of the glycerol. However, there were difficulties in using this

salt since too acid a reaction caused the sulfite to become toxic, while too basic a reaction caused the free ammonia, it was assumed, to become inhibitory. Calcium and magnesium sulfite could be readily removed as insoluble compounds from the fermentation medium, and these salts responded with somewhat higher yields of glycerol when the pH was adjusted to the correct level. This effect was more noticeable with calcium than with magnesium sulfite, perhaps because of the higher solubility of the magnesium salt. The average glycerol yield using magnesium sulfite ran about 22 to 23 per cent of the dextrose. These fermentations were run at pH levels below 7.0 or under acid conditions, thus greatly reducing the danger of contaminations.

The use of calcium sulfite and bisulfite was also reported by Duchenne (1942). A 21° Brix sirup was fermented for five days at 35° C. with S. ellipsoideus in the presence of an acid slurry of calcium sulfite and bisulfite with constant slow stirring. The fermented beer had a final pH of 4.5 and a concentration of 13 gms. of sulfur dioxide per liter. After removal of the calcium sulfite by settling, the liquor was neutralized with milk of lime, concentrated to a thick sirup and distilled with steam. The yields on sugar were 9.8 to 13.4 per cent alcohol and 10.1 to 15.3 per cent of refined glycerol, depending on the amount of sulfite used. The necessary sulfite could be obtained from the juice purification processes in certain sugar factories. A sugar factory

producing 8,000 tons of molasses per year could manufacture 6 tons of glycerol and 1,000 gallons of alcohol per day using this method, providing, of course, that it could be managed on such a large scale with profit.

The Japanese investigator, Tomoda (1921 to 1929), has conducted a great deal of work on the preparation of glycerol by fermentation. Tomoda, unlike many of the other investigators, sought by a study of the aldehyde-bisulfite complex to find means of raising the yield of glycerol. His theoretical studies led to the method of analysis used in the work for this thesis. By reducing the alkalinity of the fermenting solution the dissociation of the aldehyde complex was reduced, and Tomoda (1924) claimed that his yields were higher than either Neuberg's or Connstein and Lüdecke's. From the dissociation constants of sulfurous acid and the aldehyde-bisulfite complex (2.84×10^{-6}) Tomoda calculated that the dissociation of the complex at a pH of 6 to 8 is not appreciable, but at a pH of from 8 to 10.5 it increases to 50 per cent, and at a pH above 12 the complex is almost completely dissociated. These results were confirmed by a study of the distribution of the aldehyde between water and benzene at several different pH values. When a solution of the complex and sodium bicarbonate was distilled, all the aldehyde was distilled out of solution. By using the methylene blue technique Tomoda (1928) showed that the bisulfite ion was very injurious to yeast and that sodium sulfite and the sulfite ion arrested the propagation

of the yeast. The aldehyde-bisulfite compound itself seemed to have no harmful effects.

An interesting observation by this investigator showed that in a synthetic medium the velocity constant of the fermentation was a linear function of the number of yeast cells in a given volume, while in a natural medium such as molasses the velocity constant became a linear function of the logarithm of the number of the yeast cells. This function would hold true only within certain limits, however. In studying the effect of the sugar concentration Tomoda (1929) developed the following equation:

$$w = (a + kx)\sqrt{y},$$

where w is the amount of glycerol produced (gms. per 100 cc.), x is the initial concentration of sugar in the mash (gms. per 100 cc.), y the concentration of sulfite in the mash (as sodium bisulfite, gms. per 100 cc.), and a and k are constants. Although this equation did not check some of the results obtained in this thesis, it may well hold true for the conditions under which Tomoda worked. To explain some of his results Tomoda postulated a hypothetical zymase-sulfite complex, and from some of the results of calculations made on the basis of this hypothesis claimed that 0.2 per cent was the maximum concentration of sodium bisulfite that would permit fermentation. The presence of small amounts of 2,3-butanediol in the fermented mashes was attributed to the dissociation of the aldehyde-bisulfite complex near the end of the fermentation

and the condensation of the aldehyde followed by reduction.

There are numerous other references in the literature to the sulfite process for preparing fermentation glycerol, but most of them deal with minor variations in the processes already described. Haehn (1940) altered conditions somewhat by aerating the medium, and claimed a 21.5 per cent yield in eight hours. He also was one of the few investigators who mention controlling the pH of the medium. Haehn's medium was approximately 5 per cent sodium bisulfite, 10 per cent sugar, and one per cent yeast. Considering the cost of compressed air, there seems to be no advantage in using this aeration procedure, except that it might prevent the formation of hydrogen sulfide at the end of the fermentation, which in itself is not serious if the fermentation medium is kept acid and the fermentation has proceeded at a good rate.

Barbet (1928) introduced the novel idea of using sulfurous acid (added as sulfur dioxide) to fix the acetaldehyde instead of a fixative agent such as sodium sulfite. By this method fermentation would naturally take place at a slightly acid reaction and there would be no difficultly removable salts present. No yields were given in the patent specifications, however.

Other materials which have been used to fix the acetaldehyde are charcoal (Abderhalden, 1922), carbamic acid hydrazide (Kobel, 1928), and dimedon (Neuberg, 1922). The first of these materials removed the acetaldehyde by adsorption, the

other two by direct chemical union.

Takahashi and Asai (1933) studied the production of glycerol by 23 varieties of Mucor, this being the only reference to the production of glycerol by molds. Ordinarily the yield of glycerol was approximately 3.8 to 9.0 per cent of the sugar assimilated, but the addition of sodium bisulfite increased the amount. A yield of 21.5 per cent glycerol on sugar utilized was obtained by using sodium bisulfite in a concentration of 6 per cent.

2. The use of alkaline materials

The theoretical basis for the formation of glycerol under alkaline conditions has been mentioned previously under Neuberg's third form of fermentation. The ratio of glycerol to acetic acid follows closely the theoretical ratio derived from the third equation. Among the many alkaline substances used by this investigator (1917) were sodium carbonate, potassium carbonate, tripotassium phosphate, potassium metaborate, magnesium oxide, and zinc hydroxide. The yields of glycerol obtained by the use of alkaline substances were never as high as those obtained by the use of sodium sulfite. Adams (1919) using sodium carbonate obtained only 3 per cent glycerol. Evidently this was no improvement over an ordinary fermentation and was an exceptionally low yield for the alkaline fermentation. Connstein and Lüdecke (1919) found that they could obtain a 10 to 15 per cent yield of glycerol from

dextrose with sodium carbonate. Eoff (1918) claimed in his patent that yields of 20 to 23 per cent on the sugar could be obtained. It was claimed by Tomoda (1924) that the yields of glycerol when using sodium carbonate would not be as high as those obtained when using sodium sulfite, since the acids formed by the yeast would produce acid carbonates which would reduce the alkalinity of the medium, and that the maximum yields were obtained at a pH of about 10. The major defect of the alkaline fermentation is that the alkaline medium permits many organisms other than yeast to grow and the fermentation is damaged. Sulfite fermentations on the other hand seldom become contaminated unless the sulfite level falls too low.

During the first World War word was received in the United States Treasury Department that Germany was producing glycerol from sugar by a fermentation process. At that time the publications dealing with the sulfite processes naturally were not available. Three government laboratories started to work on the problem, and Eoff, Lindner and Beyer (1919) found that the addition of alkalies such as sodium carbonate and potassium carbonate or the bicarbonates gave rise to considerable quantities of glycerol during the fermentation of sugars. Eoff was granted a patent in 1918 which covered the production of glycerol by the fermentation of sugars in the presence of alkali. The inoculum was built up in a slightly alkaline medium; the best yeast used by this investigator seemed to be

a Saccharomyces ellipsoideus var. Steinberg. Malt sprouts and inorganic salts were used as nutrients in a dextrose medium. Other fermentable substrates used by Eoff were blackstrap molasses, cerelose or refined dextrose, corn syrup, and cane sugar. About 15 per cent sugar and approximately 5 per cent sodium carbonate were found to be the best proportions; the sodium carbonate was best added in powdered form and in successive doses. Under these conditions 20 to 25 per cent of the sugar was converted to glycerol and considerable alcohol formed, the temperature being 30° to 32° C.

In America no use was made of the Eoff process for the production of glycerol for war purposes, but Eoff conducted some small-scale fermentations at the glycerol refinery of William F. Jobbins, Incorporated, at Aurora, Illinois. Using blackstrap molasses on a 15,000 gallon scale this investigator obtained 20 to 25 per cent glycerol on the fermentable sugars. The problems in recovering the glycerol are readily understood when one realizes that an ordinary Cuban blackstrap molasses contains about 47 to 48 per cent actual sugars (52 per cent Fehling reducing material), about 8 per cent ash, 22 per cent water, and 22 per cent organic non-sugars. The latter materials together with the sodium carbonate and yeast create a very difficult mash from which to distill glycerol. The newer methods of distilling glycerol probably could handle such material, however. Of these methods the one described by Carothers, Hill and Van Natta (1933) was

especially designed for alkaline fermentation liquors. In this method the dealcoholized slops were distilled by atomizing the material and passing the spray counter current to superheated steam in a vacuum; the resulting distillate was treated with lime and was blown with air to remove phenolic compounds. Several other steps then followed to remove nitrogenous compounds and the lime, and the final product was redistilled.

McDermott, in a note for Lawrie's monograph (1928), stated that the essential step in the process for the manufacture of glycerol by the alkaline fermentation was the alteration of the course of the reaction between the enzymes of the yeast and the fermentable carbohydrates in the molasses, because of the alteration of the hydrogen-ion concentration in the medium resulting from the addition of alkali. If the fermentation was conducted at a pH of 7 to 8.8 (approximately the limits of yeast tolerance), about 35 to 40 per cent of the sugar underwent transformation according to Neuberg's third form of fermentation. The main function of the soluble ash content of the molasses in the soda ash process was to act as a buffer, preventing the decrease in pH of the medium as the fermentation proceeded for it might decrease in a synthetic medium. To improve a poor molasses it would therefore be necessary to add buffer salts or regulate the addition of sodium carbonate so as to maintain the required pH.

McDermott (1929) also patented a process for the

production of glycerol by fermentation which involved the addition of sodium carbonate.

Hickey (1941) studied the effect of controlled pH in the alkaline fermentations, using sodium carbonate and sodium hydroxide. By maintaining the pH at 7.5 with sodium carbonate, a glycerol yield of 10 per cent on sugar was obtained. When a concentrated solution of sodium hydroxide was used to control the fermentation at a pH of 8 a yield of 22.8 per cent glycerol on dextrose was obtained. In both cases solutions of the alkaline materials were used and were added automatically by the pH recording machine. Since sodium hydroxide is a strong base and can be made up in a much more concentrated solution than sodium carbonate, it was more convenient to use. This experiment also showed that it was the pH effect that was important, and not the salt concentration, as some investigators had claimed.

In connection with the alkaline fermentation Krug and McDermott (1935) introduced a novel idea by using ammonia as the alkaline agent. This had a great advantage in that no fixed salts were added to the medium, ammonium salts being readily removable. The ash content of either of the previously mentioned carbonate or sulfite processes tends to raise the boiling point of the glycerol-containing solution and, furthermore, these salts accelerate the polymerization and decomposition of the glycerol during the distillation. Still another disadvantage of the fixed salts is that they also

tend to speed the decomposition of the non-glycerol organic matter and thereby introduce difficultly removable extraneous materials into the glycerol. In the process of Krug and McDermott ammonia is added whenever the mash becomes slightly acidic (below a pH of 7.0, colorimetrically determined), the pH of the mash being adjusted from about 7.2 to 7.3. In the patent a claim is made covering the pH range from 7 to 8. Yields up to 18 per cent on sugar were obtained by using molasses and ammonia (either in solution or as a gas). Hickey (1941) also studied the effect of alkaline fermentations using ammonia. Great difficulty was found in fermenting mashes in which the pH was above 7.0 and in which an appreciable concentration on ammonium salts was present. The toxicity of either molecular ammonia or ammonium hydroxide was believed to be the hindering factor. The use of ammonium sulfite was made difficult by the fact that below a pH of 7 the toxicity of the sulfite was noticeable and above a pH of 7 the toxicity of the ammonia seemed to interfere. Ammonium ions themselves seemed to be relatively non-toxic.

Takahashi and Asai (1933) in their investigations with molds reported that a concentration of 4 per cent sodium carbonate gave a yield of 23.5 per cent glycerol on the sugar assimilated.

Much more material is to be found in the literature on the alkaline fermentation, but since there were no radical differences between these reports and those already mentioned,

they will be omitted. The reviews of Lawrie (1928) and Whalley (1942) may be referred to for further information.

The use of neutral salts has received a certain amount of attention. In Table VII are listed some of the neutral salts used by Connstein and Lüdecke (1921, 1924). The highest glycerol yield was 16 per cent. On page 138 of Lawrie's monograph a list of salts used in the production of glycerol is given. Upon examining the data it can be seen that the

Table VII

Yields of Glycerol in Fermentations Containing Various Salts.*

Salts used	Amount of salt (per cent on sugar)	Glycerol yield (per cent on sugar)
Calcium chloride	40	8.2
Ammonium chloride	30	7.3
Sodium chloride	19	8.0
Sodium sulfate	24	6.7
Sodium sulfate	48	8.0
Sodium nitrate	34	5.5
Ferrous sulfate	60	11.8
Ferrous sulfate	120	13.1
Aluminum sulfate	39	9.4
Aluminum sulfate	44	11.6
Aluminum sulfate	80	16.0

*Adapted from Lawrie (1928)

highest yields were obtained by using sodium sulfite and bisulfite; sodium carbonate ranked next, while the neutral salts seldom gave a yield above 10 per cent. The exact mechanism for the formation of glycerol in the presence of neutral salts has not been ascertained, but probably it may be Neuberg's

third form of fermentation or a reaction closely allied to it. No pH values were given in the data relative to neutral salts.

III. MATERIALS AND METHODS

A. Materials

1. Sugars

Dextrose and maltose were the two sugars used primarily in this work, except for one or two experiments wherein lactose, sucrose, and fructose were used. Most of the dextrose was of the technical anhydrous grade distributed by Pfanstiehl; some C. P. grade was also used in certain experiments. Both the technical grade of maltose hydrate containing 7 to 8 per cent of dextrins and having a specific rotation of $+125^{\circ}$ to $+135^{\circ}$, and the C. P. grade containing no dextrins and having a specific rotation of $+131^{\circ}$ were used in the preparation of several series of fermentations. The supplies of maltose were also obtained from Pfanstiehl. Other sugars used were all of the purest grades obtainable.

2. Starchy materials

Several different starchy materials were used as fermentation substrates both after acid hydrolysis and, to a certain extent, after enzyme saccharifications. The corn starch was the usual commercially available type, the moisture content averaging about 11 per cent. According to the official acid-hydrolysis method of the Association of Official Agricultural

Chemists (1940) this starch analyzed on the wet basis 92.2 per cent dextrose. In addition to the corn starch prepared by the wet-milling process, some dry-milled corn was also used. The three samples of the latter were a yellow corn meal (Quaker brand), a cream meal, and some brewer's grits. The latter two were prepared from white corn, the brewer's grits being somewhat coarser than the cream meal. The ground corn used in one or two experiments was of the ordinary yellow variety, analyzing 69.4 per cent dextrose on the wet basis by the A. O. A. C. diastatic method.

3. Saccharifying agents

The malt used in this work both to supply nutrients and as a saccharifying agent was a distiller's grade rather finely ground. The mold-bran was a sample prepared in these laboratories according to the method of Hao, Fulmer, and Underkofler (1943) from wheat bran using Aspergillus oryzae No. 38.

The acids used were of the C. P. grade. Goering (1941) discussed the advantages and disadvantages of the various mineral acids in hydrolyzing starchy materials. Since sulfuric acid is the cheapest and also one of the best, it was chosen to be used in most of this work.

4. Media used

Two different types of media were used in the various fermentations discussed in this thesis. The optimum semi-synthetic

medium developed by Hickey (1941) was used in some of the first experiments. This medium, developed for the maximum production of alcohol from dextrose by yeast and utilizing the minimum amount of added solids, has the composition shown in Table VIII.

Table VIII

Optimum Semi-synthetic Medium for Alcoholic Fermentation*.

Reagent	Weight per 100 ml. of medium
Yeast extract (Difco anhyd.)	0.375 gm.
Ammonium chloride	0.15 gm.
Dipotassium phosphate (trihydrate)	0.075 gm.
Monopotassium phosphate	0.075 gm.
Magnesium sulfate (heptahydrate)	0.02 gm.
Calcium chloride	0.01 gm.
Dextrose for carrying culture	5.0 gms.
Dextrose for inoculum	10.0 gms.
Dextrose for experimental media	15.0 gms.

*Adapted from Hickey (1941)

It was found that the 10 per cent dextrose medium was most satisfactory for the glycerol fermentation.

The above medium, although quite satisfactory, was tedious to prepare and used considerable amounts of chemicals. Since in the usual glycerol fermentation quantities of salts of various kinds are added and the yeast does not grow to any great extent a simpler medium was sought. Goering (1941) found that heavy corn steep liquor in concentrations of about 4 gms. per liter gave satisfactory alcoholic fermentations in

combination with acid-hydrolyzed starch solutions. A sugar-steep water medium was used in most of the experimental work conducted for the production of glycerol in connection with this thesis. It was found on comparing the two types of media by the alcoholic fermentation of dextrose that the steep water in conjunction with the rather hard tap water in these laboratories gave as good yields of alcohol as did the optimum semi-synthetic medium.

In many cases the actual experimental media were not sterilized since the sulfites exert an antiseptic effect which the yeast tolerates to a higher degree than other organisms. However, all of the media for carrying cultures and inocula were sterilized at 15 pounds for 15 minutes. Very few contaminations were found during the course of this work, and they were accounted for either by the failure of the yeast to ferment the sugar, as in the maltose fermentations, or by the lowering of the free sulfite at the end of the fermentations to such a level that other microorganisms could grow. In the maltose fermentations it was found that sterilization of the steep water separately was all that was necessary to keep certain sulfite-tolerant organisms from being carried into the fermentation medium. It is definitely not a good practice to sterilize any media containing sulfite since the sulfite, especially the sodium sulfite, has a strong alkaline reaction and destruction of the sugar takes place. Media other than those above mentioned which were employed for specific

purposes will be described whenever they were used.

5. Yeasts

Three different strains of yeasts were used in most of the experiments described in this thesis. A strain of Saccharomyces cerevisiae designated as No. 43 (Fleischmann's catalog No. 2.15-52) was used for many of the experiments. This yeast is a high-attenuating "top" yeast which has been found very satisfactory for alcoholic fermentations run in these laboratories for a number of years. For much of the work on the preparation of glycerol by the fermentation of maltose a strain of S. ellipsoideus var. Steinberg, American Type Culture Collection No. 4098, was used. This culture is designated as No. 53 in our laboratories. For massive inoculations Fleischmann's yeast cakes were used. These were used directly as obtained from retail stores and were found to be quite satisfactory for the fermentation of dextrose, giving reasonably reproducible results.

Yeast strains were carried on either the optimum semi-synthetic medium or the steep-water medium, the former being used in most of the earlier work. For the yeast No. 43 a 5 per cent dextrose medium was used, and for the yeast No. 53 a 5 per cent maltose solution was used. Transfers were made at intervals of two to three days, using sterile pipettes delivering a volume of one or two milliliters for inoculation. The carrying cultures, 50 ml. in volume, were kept at 30° C.

in 125-ml. Erlenmeyer flasks. All fermentations were run at 30° C. unless otherwise specified.

6. Sulfites

The sodium sulfite used was of the anhydrous reagent grade. Magnesium sulfite was obtained from both the City Chemical Company and from Mallinckrodt. The former was designated as "pure" and the latter had no grade designation. The averages for the analyses of these samples in triplicate by iodine titration in the presence of a small amount of sodium bicarbonate to prevent excessive loss of sulfur dioxide were as follows:

City Chemical product	56.5% $MgSO_3$
Mallinckrodt product	58.4% $MgSO_3$

Since $MgSO_3 \cdot 4H_2O$ contains 59.2 per cent $MgSO_3$, and $MgSO_3 \cdot 3H_2O$ contains 53.8 per cent $MgSO_3$, the analytical results indicate that the commercial products are mainly $MgSO_3 \cdot 4H_2O$. In the fermentation media containing magnesium sulfite excess solid was used; so it made little difference whether the material contained a variable amount of water of hydration or small amounts of magnesium sulfate.

B. Methods

1. pH measurements

For periodical checking of the pH of fermentation media

or for occasional readings of the pH, a Cameron pH meter was utilized. If only an approximate value of the pH was desired, as when making up carrying media, universal indicator papers were very convenient.

A Cameron pH recorder was used to observe the pH in media continuously over periods of time extending from three to as many as fifteen days. This recorder is adapted to stir the fermentation for one minute out of every four and also to control the pH of the medium by automatic addition of acid or base solution. The latter feature was described in detail by Hickey (1941). The stirring is essential when media containing magnesium sulfite are used. Both of the Cameron instruments were calibrated at pH 4 by means of a phthallate buffer, the recorder once every 24 hours, the simple meter whenever used.

Fermentations on which the pH was checked continuously by the recorder were run in a five-liter three-necked flask. Into one of the side necks the electrode assembly was inserted; the stirrer entered through the center neck and the third neck was used for removing samples and for adding base or acid to control the pH. It was found necessary to take one very special precaution when the recorder was used; this was to ground every metallic part in the region of the electrodes. If this were not done, the pH recording was frequently extremely erratic.

2. Alcohol determination

All determinations of alcohol were made by distilling either the whole medium or an aliquot, usually 300 ml., from a Kjeldahl flask and collecting 100 ml. of distillate in a volumetric flask. A little solid calcium carbonate was added before distillation to neutralize any acids present in the medium, except in the case of the sodium sulfite fermentations where no calcium carbonate was used. The distillates from the sodium sulfite media were redistilled from a flask containing 5 gms. of a mixture of 3 parts of sodium sulfite and one part of sodium bisulfite. In this way the amount of aldehyde carried over was reduced to a mere trace. The sulfite holds back a certain amount of the aldehyde, and the rest is probably lost by volatilization during the two distillations. The final distillate in each case was brought to a temperature of 25° C. and the specific gravity was determined by means of a chainomatic Westphal balance. A few drops of lard oil added to the medium to be distilled aided in retarding foam formation.

3. Sugar determination

Reducing sugars were determined by a modified Shaffer-Somogyi method as described by Underkofler, Guymon, Rayman and Fulmer (1943). This method is quite satisfactory for media not containing sulfite. If sulfite is present, it must be completely removed or it will interfere in the final

titration with sodium thiosulfate, since the sulfite will reduce the iodine liberated from the potassium iodate, thus giving high sugar values. The removal of the sulfite was ordinarily accomplished by boiling an aliquot sample of the medium, usually 5 ml., with acid and then precipitating with basic lead acetate. Whenever the soluble sulfite was very high, however, this procedure was found to be unreliable, and in most of the sulfite fermentations the final sugar values were not determined.

Optical rotations were employed in several cases to get an approximate idea of the amount of sugar left in the fermented beers, but these measurements are complicated by the presence of materials other than sugar and the uncertainty in the case of maltose as to whether the yeast has converted any of the sugar to dextrose. In any case since the aim of the work was to get the highest possible yield of glycerol based on the initial sugar the determination of residual sugar was not a vital matter.

4. Glycerol and acetaldehyde determinations

One method which may be employed in the determination of glycerol formed in fermentations is that developed by Fulmer, Hickey and Underkofler (1940). This method involves the determination of the residual sugar by the procedure mentioned previously and then oxidizing the glycerol and sugar in a separate sample by means of ceric sulfate. By means of

standard curves the amount of ceric sulfate needed to oxidize the glycerol is found and from this value the amount of glycerol determined. The presence of proteins, most of which can be removed by basic lead acetate, sulfite, or any other oxidizable material, interferes with the analysis. Whenever the medium is of the semi-synthetic type and only small amounts of soluble sulfite or sodium salts are present, the sample may be treated with acid, boiled, and then clarified with lead acetate. The results obtained by the ceric sulfate oxidation method then are fairly reliable. However, in the presence of high concentrations of sulfite, especially sodium sulfite, the method becomes unsatisfactory even when great care is taken to remove interfering materials. None of the determinations of glycerol described in this thesis were made by the ceric sulfate-oxidation method, although many such were run in earlier unreported work. Several other oxidation methods for the determination of glycerol are described in the literature, such as the periodic acid method of Bradford (1942) and also the method of Smith and Duke (1943).

The method employed for the determination of glycerol in the fermentations described in this thesis was an adaptation of a method described by Tomoda (1929). By determining the amount of bisulfite fixed by the acetaldehyde during the fermentation the amount of glycerol can be calculated. The theoretical basis of the determination of the sulfite combined with the acetaldehyde is founded on the fact that the

aldehyde-bisulfite complex is only very slightly dissociated in acid solution (pH 5 to 7), but as the solution is made alkaline (pH 8) the dissociation of the complex increases to such an extent that all of the bound sulfite can be titrated with iodine solution.

Ripper (1900) utilized a method somewhat similar to that later developed by Tomoda for the determination of acetaldehyde. In Ripper's method the acetaldehyde is distilled into a solution of potassium bisulfite and the amount of free sulfite is determined by iodine titration. The difference between the original amount of sulfite and the free sulfite is equal to the amount of bound sulfite which in turn is equivalent to the acetaldehyde present. The advantage in Tomoda's method lies in the fact that no distillation is necessary thus avoiding any manipulative losses of the aldehyde during the distillation. Another distinct advantage is that the total amount of sulfite present has little or no effect upon the analysis. Tomoda when checking his method against that developed by Ripper found that his results were slightly higher than the latter's, which might be expected in view of the possible negative errors in Ripper's method.

The equivalence between acetaldehyde and glycerol has been investigated by several workers and all are agreed that the theoretical relationship is correct. Since many of the early investigators used Ripper's method for the determination of acetaldehyde, and recovery methods followed by

oxidations to determine the glycerol, very few of the workers obtained exactly the ratio of 2.09 as demanded by the equation:

$$C_6H_{12}O_6 \longrightarrow C_3H_8O_3 + CH_3CHO + CO_2.$$

In the calculations employed for this thesis the theoretical ratio was used.

The details of the analytical method actually employed are given in the following paragraphs.

Reagents:

A. 0.1 N iodine solution containing 12.7 gms. resublimed iodine and 16.0 gms. potassium iodide, reagent grade, made up to one liter and standardized against sodium thiosulfate solution of known normality.

B. One per cent starch solution, made up in saturated sodium chloride solution to prevent the growth of microörganisms.

C. Sodium bicarbonate, reagent grade.

Five milliliters of the fermentation medium to be analyzed is measured into a 125-ml. Erlenmeyer flask and 0.5 ml. of the starch solution is added. The sides of the flask are washed down with distilled water, and the titration with iodine is carried out until the first purple color that persists more than fifteen seconds appears. If the endpoint is overstepped, back titration with a 0.1 N sodium thiosulfate solution may be resorted to in order to rectify the error. The volume of iodine consumed in the first titration is equivalent to the free or unbound sulfite. Solid sodium bicarbonate is then added until some solid remains undissolved

and the color of the starch-iodine complex disappears. The titration with iodine is then continued and the volume of iodine consumed in this second titration is equivalent to the amount of sulfite bound by the acetaldehyde. Quite often in the first titration with iodine the endpoint is rather fleeting when the amount of free sulfite falls much below the equivalent of 5 ml. of iodine. The addition of 0.5 ml. of 6 N hydrochloric acid before titration will remedy this. One difficulty often encountered is excessive foaming when the bicarbonate is added, but the foam is easily collapsed by the addition of a drop or two of Aerosol solution. The iodine solution is best delivered from an automatic burette such as the pressure-filling automatic overflow burette sold by the Ace Glass Company (catalog No. 5750). This particular burette has an advantage in that the iodine solution does not come in contact with rubber.

The volume of iodine consumed in the second titration for the bound sulfite is corrected to 0.1 N if the iodine solution is not of that normality and then the amount of glycerol may be calculated from the relationship:

$$\text{ml. of 0.1 N } I_2 \times 0.0046 = \text{gms. glycerol.}$$

The amount of glycerol is then multiplied by a volume factor to obtain the total amount of glycerol present in the medium. For instance if the volume of the medium is 310 ml. the volume factor would be 62 since the volume of the sample

titrated is 5 ml. If magnesium sulfite is used as the aldehyde fixing agent, the medium should be centrifuged and 5 ml. of the centrifugate used for the analysis. In this way the actual amount of sulfite dissolved in the medium may be determined, which would not be the case if a variable amount of the solid sulfite is present to react with the iodine.

There will naturally always be a slight difference between the amount of glycerol as determined by the above method and the amount actually present in the medium. A certain amount of glycerol is usually present in the medium which cannot be accounted for by Neuberg's second form of fermentation. This glycerol may be the "protein-glycerol" as described by Gehle (1922) or that formed in the initial phases of the fermentation according to the Embden-Meyerhof-Parnas scheme. The acetaldehyde-bisulfite complex may also dissociate slightly to liberate some aldehyde which in turn may be reduced to alcohol or converted to 2,3-butanediol by the yeast. In any case the errors are small and are negative errors, so that the yields of glycerol obtained by the iodine-titration method may possibly be a little low. The error within a given series of fermentations should be constant and therefore the trend of the yields should be unaffected. In a private communication Dr. R. J. Hickey (1943) stated that a comparison of the iodine-titration method with a recovery method and with the ceric sulfate oxidation method showed that the amount of glycerol found by actual recovery agreed with the values

obtained by the iodine-titration method, but that the values obtained by the oxidation method were too high. The lack of interfering substances and the rapidity of the analysis are two of the best features of the modified method of Tomoda. For the purposes of this thesis the advantages of the method far outweigh the disadvantages.

Recovery methods are slow and time-consuming and, furthermore, are subject to innumerable errors. Lawrie (1928) describes several recovery methods for the determination of glycerol present in fermentation media. Most of these methods require a further step, usually an oxidation, to determine the purity of the glycerol recovered. During the present investigation the acetone recovery method described by Lawrie was carried out in duplicate on a fermented medium which gave a value of 29.2 per cent glycerol on dextrose by the iodine-titration method. As an extra precaution sodium bicarbonate was added to the 100 ml. sample before evaporation in order to decompose the aldehyde-bisulfite complex. The sample was evaporated to almost complete dryness and then excess solid anhydrous sodium sulfate was added. The dry mass thus obtained was extracted for twenty-four hours with acetone in a Soxhlet extractor, the acetone removed by distillation, and the residue dissolved in absolute alcohol. The alcohol solution was centrifuged, the alcohol evaporated from the centrifugate, and the residue was again dissolved in absolute alcohol and the process repeated. The residual material

obtained from the last evaporation was dried in an oven at 110° C. until the weight loss was about 0.02 gms. for a fifteen-minute period of heating. The material obtained was definitely impure since oily material could be plainly seen in the sample and the "glycerol" was a very dark brown. The per cent glycerol on dextrose obtained by this method amounted to 36 per cent, which was evidently too high.

Using the same fermented material as employed for the acetone recovery method, four samples were treated in the following manner. One hundred milliliters of the fermented beer was evaporated to a thick syrup in the presence of 5 gms. of sodium bicarbonate. The complex of acetaldehyde and bisulfite must be completely destroyed by the bicarbonate in this step or the recovery will be considerably complicated. The evaporated material was treated with absolute alcohol and then to the filtered alcohol extract an equal volume of anhydrous ethyl ether was added. The resulting suspension was filtered, and the solvents evaporated on a steam bath. The resulting viscous material was extracted with a fifty-fifty alcohol-ether mixture, filtered, and the solvents again evaporated. The final drying was carried out in an oven as described previously. The results obtained checked very well with the value of 29.2 per cent obtained by the iodine-titration method. The results were 31.0, 29.5, 29.7, and 27.8 (average - 29.5) per cent. The material obtained by the above recovery method was a clear, light yellow viscous

product which certainly should be free from moisture. This recovery method, although checking quite well with the modified method of Tomoda, is time-consuming and subject to errors arising from the various transfers.

There are many other methods for the determination of glycerol in fermentation media but they need not be described here. Descriptions may be found in the reviews of Lawrie (1928) and Whalley (1942). It may be noted here that, unless otherwise stated, all glycerol yields reported in this thesis are expressed on the basis of per cent product on initial substrate.

5. Yeast cell counts

Cell counts were made by using a Thoma-Zeiss haemocytometer. The Thoma-Zeiss counting cell is divided into sixteen large squares each of which is subdivided into sixteen smaller squares. One yeast cell per large square is equivalent to 250,000 yeast cells per milliliter of medium. In making cell counts the number of yeast cells present in ten of the larger squares was counted and the average value was taken as the count for one large square. For purposes of simplification the counts will be given in this thesis as the average number of cells per large square and not as the total number of cells per milliliter. When excessive numbers of cells were present the medium was diluted to facilitate the counting. If excessive clumping of the yeast occurs, accurate counting is almost impossible.

6. Moisture content of starchy materials

A very simple and reasonably accurate method was adopted for this determination. A weighed aluminum dish, approximately 3 inches in diameter and 0.5 inch deep, was filled to a depth of about 0.25 inch with the material to be tested and was again weighed. The dish and its contents were placed in an oven at 110° C. for about twelve hours, cooled in a desiccator, and weighed. The loss in weight represented the amount of moisture originally present in the material. Weighings were carried out to the nearest centigram, and the results checked to within 0.1 of one per cent.

IV. EXPERIMENTAL

A. The Use of Enzyme-Converted Starchy Materials as Sources of Fermentable Sugars

There are two general methods for converting starchy materials such as corn or corn starch to sugars fermentable by yeast. One method employs enzymes usually obtained from sprouted barley grain, commonly called malt, or from mold-bran to carry out the conversion, the other method involves the use of mineral acids and increased temperatures to bring about the hydrolysis of the starch. Malt converts the starch molecule to maltose primarily; mold-bran converts the starch to maltose and probably some dextrose; acids convert the greater part of the starch to dextrose although maltose and dextrins can be found in the hydrolyzate in various amounts depending on the severity of the treatment.

Goering (1941) obtained almost complete conversion of a 7.5 per cent starch suspension to fermentable sugars by hydrolyzing the suspension in the presence of 0.02 N sulfuric acid under 25 pounds steam pressure for four hours. His work also indicated that higher starch concentrations required higher acid concentrations, and that as good alcohol yields were not obtained under these conditions as when lower starch concentrations were used. Ground corn also required higher acid

concentrations than did corn starch. When ground corn was acid-hydrolyzed some toxic factor seemed to be present which interfered with the metabolism of the yeast. It was demonstrated that this factor probably arose from the bran. Actually, per pound of fermentable sugar obtained, starch is not much more expensive than corn itself.

As has been mentioned previously, very few references are to be found on the use of starchy materials as sources of fermentable sugars to be used in the preparation of glycerol by fermentation, despite the fact that several patents claim their use. Eoff (1919) is the only investigator who mentions actually using a corn syrup as a source of fermentable sugar. The experiments described in this and in the following sections were made in an attempt to determine the feasibility of using starchy materials as sources of fermentable sugars.

1. The use of a malted corn mash as a source of fermentable sugar

Several preliminary fermentations were run using malted corn mashes containing magnesium sulfite as the aldehyde-fixing agent. The results of these experiments were very unsatisfactory; in fact, yields of glycerol of more than 6 per cent of the reducing sugar, calculated as maltose, were rarely obtained. One thing was noted, however, namely that long periods of saccharification seemed to be necessary, perhaps because the presence of sulfite inhibited the action of the malt enzymes.

In one of the better fermentations the procedure used was as follows. Two hundred grams of ground corn was suspended in one liter of 0.04 N hydrochloric acid, steamed for fifteen minutes, and then autoclaved at 15 pounds pressure for one-half hour. The resulting mash was cooled to 63° C. and 20 gms. of malt was added. The flask containing the mash was immersed in the malting bath, the temperature of which was maintained at 53° C. and 10 gms. more of malt was added after eighteen hours and again after twenty-four hours. After about thirty hours the mash was filtered and 650 ml. of filtrate was obtained which gave only a very faint iodine-starch test and had a pH of about 6. By analysis 9.44 gms. of maltose hydrate was present per 100 ml. of filtrate.

The filtrate was divided into three portions, two of 250 ml. and the third of 150 ml. To the two larger portions 20 gms. of magnesium sulfite was added, and then all three media were inoculated with 20 ml. of a thirty-six-hour inoculum of yeast No. 43 grown on a 5 per cent dextrose-steep water medium. Twenty-four hours later the media containing the magnesium sulfite were just beginning to gas and had a pH of 6.8 while the medium containing no magnesium sulfite had been gassing vigorously for some time. After a week had passed, the formation of gas, which had never been very rapid, seemed to be slowing down considerably in the media containing magnesium sulfite, and the media were analyzed for glycerol by the iodine-titration method. The average yield of glycerol

from maltose was 15.4 per cent, which was considerably better than had been found in some of the preliminary work. In view of some of the later work the pH of 6.8 may have been responsible for the better than average yields.

Another malt-saccharified fermentation was prepared by using corn starch instead of ground corn. The major difference between this and the preceding fermentation was that acid-thinning was not resorted to, and, as a result, the pH of the medium after the magnesium sulfite had been added was 8.0. The sugar concentration was somewhat lower, being 6.4 gms. of maltose hydrate per 100 ml. Eighty-eight hours after inoculation only 1.5 ml. of iodine was required for the fixed sulfite, and the mash began to develop a noticeably bad odor. Microscopic examination revealed the presence of a short rod-like contaminant which was not further identified. Later work with maltose showed that whenever the medium had a pH of 7.2 or higher fermentation by yeast seldom took place and contaminants began to grow.

2. pH recording of the fermentation of a malted corn mash in the presence of magnesium sulfite

In order to get an idea of the rate of formation of glycerol and the trend of the pH during the fermentation of a malted corn mash in the presence of magnesium sulfite, a larger amount of medium was needed. This was prepared by combining 2000 gms. of ground corn with 9 liters of tap water

and then adding 120 gms. of malt. The combined mash was held at 63° C. for one-half hour, gelatinized by heating, and then cooked under 5 pounds pressure for one and one-half hours. The rather thick mash was diluted to about 16 liters, cooled to 63° C., and 1300 gms. of malt was added. This is a very high proportion of malt, but a rather complete conversion was desired in as short a time as possible. Twelve hours later the mash was filtered and 3800 ml. of the filtrate reserved for the fermentation. To serve as inoculum 400 ml. of this filtrate was inoculated with yeast No. 43 and about 30 gms. of magnesium sulfite was added. The remaining 3400 ml. of mash was autoclaved for thirty minutes under 15 pounds pressure. The analysis of the autoclaved filtrate showed the presence of 11.4 gms. of maltose hydrate per 100 ml.

After the inoculum had grown forty-eight hours it was filtered with the aid of a small amount of Celite and the filter cake added to the 3400-ml. portion of mash. Ten milliliters of steep water and 300 gms. of magnesium sulfite were also added. The fermentation was carried out in a 5-liter, 3-necked flask equipped with a stirrer controlled by the automatic timer in the pH recorder which automatically stirred the fermentation every four minutes. In Table IX the pH values and the analytical data obtained from periodical analyses of the fermenting medium are given.

Table IX

Fermentation of a Malted Corn Mash in the Presence of Magnesium Sulfite

Age (hrs.)	pH	Ml. of 0.1 N. Iodine per 5 ml. of mash		
		Free sulfite	Fixed sulfite	Total sulfite
4	7.0	18.1	0.5	18.6
24	6.4	17.1	4.7	21.8
48	6.5	15.1	6.7	21.8
72	6.6	13.3	8.5	21.8
96	6.5	12.8	8.9	21.7
120	6.6	11.8	9.7	21.5
144	6.6	10.4	11.4	21.8
168	6.6	8.6	12.6	21.2
192	6.6	6.7	14.1	20.8
216	6.6	5.3	15.3	20.6
240*	6.6	4.8	15.5	20.3
264	6.6	4.7	15.6	20.3

*Ten days

Around the tenth day of fermentation signs of contamination were noticed, and since the fermentation seemed to be finished, the recording was stopped. At acid pH levels contamination is seldom encountered in the sulfite fermentations unless, as it did in this case, the free sulfite level falls below the equivalent of 5 ml. of iodine per 5-ml. sample. It is also worthy of note that the amount of total sulfite present at any time during the fermentation is dependent to a greater degree on the pH of the medium than on the amount of fixed sulfite. The analysis for the eleventh day showed the presence of 48.8 gms. of glycerol in the total mash, and this

is equivalent to a yield of 12.0 per cent on the original maltose hydrate present. It would also be 12.0 per cent on the dextrose equivalent of the maltose since the molecular weight of maltose hydrate is exactly the same as the sum of the molecular weights of the two dextrose molecules that could be obtained from it by hydrolysis.

3. The fermentation of acid-thinned starchy materials saccharified by malt and mold-bran

The same general procedure was used in preparing flasks Nos. 1 to 5 in this experiment although they were made up at different times. Thirty grams (26.7 gms. dry basis) of the starchy material to be used was mixed with 300 ml. of 0.04 N hydrochloric acid, gelatinized, and cooked for one hour at 15 pounds steam pressure. To the cooked mash about one gm. of calcium carbonate was added to neutralize the acid, and after the mash had cooled to the proper temperature, the enzyme-containing material was added. For the saccharifications with malt 1.8 gms. of a good grade distiller's malt was added, and the mash held at 55° C. for five hours; for the mold-bran saccharifications 1.8 gms. of a mold-bran prepared with A. oryzae was added and the mash held at 30° C. for about two hours. The pH of the mashes during saccharification was about 5.0. After the saccharification period 25 gms. of magnesium sulfite was added along with 30 ml. of a 30-hr. culture of yeast No. 43 grown on a 5-per cent dextrose-steep water

medium. The flasks were shaken at intervals to insure good contact between the sulfite and the medium. All of the media were made up in duplicate and were analyzed at the end of five days.

A larger batch was made up to be run on the recorder. Three hundred grams of cream meal was mixed with 3 liters of 0.04 N hydrochloric acid, gelatinized, and cooked for one hour at 15 pounds pressure. When the resulting mash had cooled down the recorder electrode assembly was immersed in the mash and 10 gms. of calcium carbonate added, whereupon the pH fell to 5.0. At this point 18 gms. of mold-bran was added and the mash held at the incubator temperature of 30° C. for one and one-half hours with intermittent stirring. Then 300 gms. of magnesium sulfite was added along with 310 ml. of a 30-hr. inoculum of yeast No. 43 grown on a 5-per cent dextrose-steep water medium, and the pH immediately rose to 7.5. In twenty hours the pH had fallen to 6.5, and it remained in that region to the end of the fermentation. The amount of fixed sulfite rose slowly and apparently stopped increasing on the sixth day, at which time 5 ml. of mash required 9.7 ml. of 0.1 N iodine for the fixed sulfite. The analysis of this fermentation (No. 6) is given in Table X along with the analysis of the preceding fermentations (Nos. 1 to 5).

Table X

Fermentation of Enzyme-Saccharified, Acid-Thinned Starchy Materials in the Presence of Magnesium Sulfite.

Flask No.	Starchy Material	Enzyme Source	Yeast Strain (No.)	Glycerol yield* (%)
1	Corn starch	Mold-bran	43	13.2
2	Yellow corn meal	Mold-bran	43	10.5
3	Yellow corn meal	Malt	43	11.2
4	Cream meal	Mold-bran	53	12.9
5	Cream meal	Malt	53	12.6
6	Cream meal	Mold-bran	43	11.5

*The yield of glycerol is calculated as per cent of dry starchy material converted to glycerol.

Although the yields of glycerol were calculated on the basis of dry starchy material, yields were still in the range of 12 per cent, which seems to be the highest obtainable from enzyme-saccharified materials for a fermentation of four to five days' duration. On examining the data of Table X, no great difference can be seen in the use of mold-bran or malt, but yeast strain No. 53 seems to be somewhat better than No. 43 for these conditions.

4. The fermentation of a malted corn mash in the presence of various salts

The reason for the poor utilization of the maltose in the previous fermentations might be ascribed to the specific

effect of the magnesium or sulfite ions, to the salt concentration, or perhaps to the removal of phosphate from the medium by the magnesium ion. In order to get an idea as to what might be the hindering factor, a malted corn mash was prepared in a similar manner to the preparation of the mash in experiment 2 of this section. Two hundred gms. of ground corn, 4 gms. of malt, and 900 ml. of tap water were maintained at 60° C. for one-half hour and then gelatinized and cooked at 12 lbs. pressure for one-half hour. The resulting mash was cooled to about 60° C., and a slurry of 130 gms. of malt in 600 ml. of water was added. After allowing the saccharification to proceed for about three hours at 55° C. the mash was filtered. The resulting filtrate totaled 1250 ml. and a sample gave a rotation of +20.3° in a 2-dm. tube using the yellow light of sodium. The filtrate was divided between four flasks in 300-ml. portions, and each flask was then inoculated with 30 ml. of a 24-hr. culture of yeast No. 43 grown on a 5-per cent maltose-steep water medium. Two hours later, to separate flasks various additions of salts were made, the additions being such as to make the concentration about 0.25 M, approximately the concentration of soluble magnesium sulfite, when this salt is used, in the medium. After three days the angles of rotation were measured for the medium in each flask under the same conditions as the rotation of the original filtrate was measured. The results are shown in Table XI.

Table XI

Fermentation of a Malted Corn Mash in the Presence of Various Salts.

Salt addition	α_D^{20} for 2 dm.*
10 gms. $MgSO_3$	+17.2°
10 gms. $MgSO_3$ + 1 gm. $K_2HPO_4 \cdot 3H_2O$	+17.6°
9 gms. $MgSO_4$	+5.5°
11 gms. Na_2SO_4	+5.6°

*Rotation of original sugar solution was +20.3°.

No calculations were made as to the actual concentrations of maltose present since the presence of the salts would introduce uncertainty into the determination. The presence of the two soluble salts only slowed up the fermentation since over two-thirds of the original sugar had been fermented according to these data. Stark and Somogyi (1942) indicated that electrolytes exert a slight inhibitory effect on the fermentation of maltose. However, the presence of magnesium sulfite definitely hindered the utilization of the sugar. Since the hindrance could not have been caused by the magnesium ion or by the salt concentration and probably not by the lack of phosphate; the sulfite ion, or perhaps the bisulfite ion, evidently was the cause of the poor fermentations. Iodine titration of the two flasks containing the magnesium sulfite showed that only about 2 per cent of the maltose had been converted into glycerol; or assuming from the rotation data that one gram of maltose had been fermented, about 14 per cent

glycerol was produced from the utilized sugar. Since yeast ferments dextrose satisfactorily in the presence of magnesium sulfite, it is apparent that in some way sulfite hinders the fermentation of maltose.

B. The Fermentation of Acid-Hydrolyzed Starchy Materials in the Presence of Magnesium Sulfite

1. The fermentability of acid-hydrolyzed corn starch

In order to obtain an idea as to the fermentability of acid-hydrolyzed starch two samples of 22.5 gms. (wet basis) of corn starch were weighed into separate 500-ml. Erlenmeyer flasks, and to each flask 300 ml. of 0.02 N sulfuric acid was added. The flasks were heated for four hours at 25 lbs. steam pressure. After this hydrolysis the media in the flasks were dark brown and contained some suspended solid material. Five milliliters of medium was removed from each flask and analyzed for reducing sugar; flasks A and B respectively contained 6.21 and 5.84 gms. per 100 ml. of reducing sugar calculated as dextrose. These values corresponded to a conversion of starch to dextrose of 84.5 and 79.5 per cent of theoretical, respectively. Higher conversions probably would have been obtained if the starch had been gelatinized before cooking. To flask A 25 gms. of magnesium sulfite and one milliliter of steep water were added, and the pH adjusted to 6.0. Flask B was adjusted to pH 5 with sodium carbonate, and then both flasks

were inoculated with 20 ml. of a 24-hr. culture of yeast No. 43 grown on a 5 per cent dextrose-steep water medium. After six days the flasks were analyzed, although analysis could have been run sooner since all evidence of gassing ceased on the fourth day.

Flask B showed the presence of only 0.1 gms. of dextrose per 100 ml., indicating that fermentation had been practically complete. By iodine titration flask A showed the presence of a total of 4.31 gms. of glycerol which would correspond to a yield of 25.0 per cent on reducing sugar or 22.1 per cent on original dry starch. Evidently from these data acid-hydrolyzed starch is quite satisfactory as a fermentable substrate.

2. pH recording of the fermentation of acid-hydrolyzed starch

The first fermentation of this type fermented satisfactorily, but, because of faulty operation of the recording machine, the trend in the pH was difficult to determine. However, at the end of about 55 hours iodine titration showed the fermentation had ceased and that about 25.0 per cent of the dry starch had been converted to glycerol.

Another medium was made up by suspending 225 gms. of starch in three liters of 0.02 N sulfuric acid, gelatinizing the suspension over a burner, and then cooking it for four hours under 25 lbs. pressure. To the resulting light yellow hydrolyzate calcium carbonate was added to neutralize the acid, and the medium was filtered. By analysis the medium

contained 6.89 gms. of dextrose per 100 ml., the total volume being 3050 ml. The conversion of the starch was practically quantitative. A culture of No. 43 yeast grown in 300 ml. of a 5-per cent dextrose-steep water medium containing 15 gms. of magnesium sulfite was filtered and the filter-cake was used as the inoculum. To furnish nutrients, 15 ml. of steep water was added, and then 300 gms. of magnesium sulfite was added. The automatic periodical stirring kept the sulfite in good contact with the medium. At intervals samples were removed and analyzed by the iodine titration method for fixed sulfite. The results are shown in Table XII.

Table XII

pH Recording of the Fermentation of Acid-Hydrolyzed Starch in the Presence of Magnesium Sulfite.

Age (hrs.)	pH	Ml. of 0.1 N iodine per 5-ml. sample	
		Free sulfite	Fixed sulfite
2	7.9	24.0	0.7
19	6.8	20.1	6.2
27	6.7	16.2	9.2
41	6.8	12.5	17.1
49	7.0	11.4	17.6
65	7.5	11.5	17.9
89	7.7	(10.4)	(15.6)
113	7.8	11.8	16.1
137	7.8	11.4	15.8

From these data it is apparent that the fermentation reached completion in about sixty-five hours and the maximum

amount of iodine required for the fixed sulfite was 17.9 ml. (for a 5-ml. sample). This would be equivalent to a yield of 25.3 per cent glycerol formed from the dry starch or 23.9 per cent based on reducing sugars.

Characteristically the acidity of the fermentation rose to a maximum as the fermentation proceeded, and then, as the fermentation neared its end, the acidity began to fall and the pH rose rapidly. The slight drop in the amount of the fixed sulfite after the peak has been reached is also quite characteristic of magnesium sulfite fermentations. The gradual dissociation of the aldehyde-bisulfite complex and the subsequent reduction of the acetaldehyde by the yeast might account for this decrease.

An attempt was made to run another fermentation similar to the one just described but at a controlled pH of 6.6. Unfortunately the control mechanism added too much acid at one stage, and the pH fell to 5.6. The change was discovered and altered several hours later, but the fermentation was dead. It had been noted in previous fermentations employing magnesium sulfite that whenever the pH falls below 6.0 to 6.1 the medium becomes definitely toxic to the yeast. ?

3. Fermentation of acid-hydrolyzed starch in the presence of magnesium sulfite at pH 6.3

This fermentation was run in conjunction with the automatic pH control mechanism of the recorder, and the acidity was

maintained at pH 6.3 by the addition of a 1:1 dilution of hydrochloric acid. The medium was prepared by suspending 300 gms. of starch in three liters of 0.05 N sulfuric acid, gelatinizing the suspension over a burner, and then cooking for four hours under 25 lbs. pressure. The resulting medium contained 8.85 gms. of dextrose per 100 ml., or a total of 277 gms. of dextrose, which represents a quantitative conversion of starch to dextrose. A quantitative conversion actually only means that as much dextrose was obtained from the starch as was obtained by using the acid-hydrolysis method of the A. O. A. C., and the reducing sugar may not all be dextrose, but is calculated as such. The recorder electrode assembly was then immersed in the medium, and calcium carbonate was added, bringing the pH up to 5.1. Three hundred grams of magnesium sulfite was then added, whereupon the pH rose to 8.0, but was lowered to 6.8 by the hydrochloric acid. At this point a filter cake obtained by filtering a 27-hr. culture of yeast No. 43 grown in 300 ml. of a 5-per cent dextrose-steep water medium containing magnesium sulfite was added. The pH was gradually lowered to 6.3 and maintained there during the course of the fermentation. Samples were removed at intervals and analyzed, the results of the analyses being shown in Table XIII.

It may be noticed in this table that the total sulfite values did not decrease as is common in uncontrolled fermentations where the pH rises at the completion of the

fermentation, but rather the value for the total sulfite rose slightly, indicating that there was a shift in the equilibrium between the four components of this system: magnesium sulfite (solid), magnesium sulfite (dissolved), magnesium bisulfite, and the aldehyde-bisulfite compound.

Table XIII

Fermentation of Acid-Hydrolyzed Starch in the Presence of Magnesium Sulfite at pH 6.3.

Age (hrs.)	Ml. of 0.1 N iodine per 5-ml. sample		
	Free sulfite	Fixed sulfite	Total sulfite
2	20.2	1.1	21.3
20	19.4	1.9	21.3
31	18.0	4.2	22.2
43	15.8	6.7	22.5
54	13.7	9.0	22.7
64	13.9	9.9	23.8
78	12.1	11.9	24.0
102	9.7	14.4	24.1
126	8.0	16.6	24.6
150	10.7	17.1	27.8
174	10.2	17.2	27.4

After one hundred seventy-four hours, the fermentation appeared to be completed, and the maximum value of iodine fixed was equivalent to 52.1 gms. of glycerol in the whole medium which totaled 3295 ml. This would correspond to a yield of 18.8 per cent glycerol on dextrose or 19.5 per cent on dry starch. About 50 ml. of acid was required to maintain the pH at 6.3 during the course of the fermentation. The

yield of glycerol obtained in this fermentation was lower than that obtained in the previous one and proceeded much more slowly, taking almost twice as long to reach completion. Evidently it would be better to let a fermentation of this type ferment without pH control or perhaps control it at a slightly higher pH in the neighborhood of 6.6 to 6.7.

4. The fermentation of starchy materials hydrolyzed by 0.02 N sulfuric acid

In this series four different starchy materials were used, namely cream meal, brewer's grits, yellow corn meal, and corn starch containing 10.9, 10.8, 11.1, and 11.1 per cent moisture respectively. Sixty grams of each material was weighed into separate one-liter Erlenmeyer flasks and to each flask 600 ml. of 0.02 N sulfuric acid was added. The mashes were gelatinized over a burner and cooked for four hours at 25 lbs. steam pressure. The corn starch medium showed a pH of 2.0 while the other mashes had a pH of 4 and also contained considerable solid material. The mashes were neutralized with calcium carbonate and filtered, and then each filtrate was divided into two equal portions. The results of the sugar analyses are given in Table XIV.

Sherman (1935) states that new-process corn meal (yellow) contains 78 per cent carbohydrates, calculated as dextrose, exclusive of fibers, so it is apparent that the conversion of the dry-milled meals was definitely not complete.

Table XIV

Sugar Content of Mashes Containing Starchy Materials Hydrolyzed by 0.02 N Sulfuric Acid.

Starchy material	Volume (ml.)	Dextrose gms. per 100 ml.	Total dextrose in mash (gms.)	Total* dextrose formed (gms.)	Per cent conversion
Cream meal	595	2.92	17.4	18.1	33.8
Brewer's grits	595	2.92	17.4	18.1	33.8
Yellow corn meal	600	2.51	15.1	15.6	29.3
Corn starch	620	7.67	47.6	47.6	89.5

*Calculated on the basis of a volume of 620 ml. since considerable liquid was retained by the solids except in the case of corn starch where only a negligible amount of solid remained.

To each portion of the filtrates 20 gms. of magnesium sulfite, two ml. of steep water, and 30 ml. of a 30-hr. culture of yeast No. 43 were added. The flasks were agitated at intervals, and at the end of five days the fermented mashes were analyzed. The results of the analyses are shown in Table XV.

The yields of glycerol from the starchy materials are lower than the yields calculated on the basis of the dextrose present in the medium because the conversion of starch to dextrose was very poor. The yields from corn starch were definitely better than those from the dry-milled meals. The presence of reducing materials which may not be fermentable may account for the low yields computed on the basis of reducing sugars calculated as dextrose.

Table XV

Fermentation of Acid-Hydrolyzed Starchy Materials in the Presence of Magnesium Sulfite.

Starchy material	Glycerol formed (gms.)	Gms. dextrose	Gms. dry starchy material	% glycerol formed from	
				Dextrose	Starchy material
Cream meal	1.09	8.7	26.8	12.5	4.1
Brewer's grits	1.02	8.7	26.8	11.8	3.8
Yellow corn meal	0.91	7.6	26.7	12.0	3.4
Corn starch	4.51	23.8	26.7	19.0	16.9

5. The fermentation of starchy materials hydrolyzed by various concentrations of sulfuric acid

Three different starchy materials were mixed with varying concentrations of sulfuric acid. The various combinations, along with the numbers employed on various flasks, were as follows:

	Acid concentration		
	0.05 N	0.1 N	0.2 N
Corn starch	S 5	S 1	---
Yellow corn meal	Y 5	Y 1	Y 2
Cream meal	C 5	C 1	C 2

All mashes were prepared in duplicate by adding 300 ml. of the acid to 30 gms. of the starchy materials in a 500-ml. Erlenmeyer flask. The mashes were then gelatinized over a burner and cooked for four hours at 25 lbs. pressure.

The average values for the amount of dextrose in each pair of flasks are given in Table XVI. The total amount of dextrose was calculated on the basis of a total volume of 310 ml., while the amount of dextrose in the filtrate was calculated on the basis of the actual volume obtained after each flask had been treated with calcium carbonate and filtered. A dextrose content of 77.3 per cent was found for the yellow corn meal (wet basis), which value agrees rather well with Sherman's value of 78.5 per cent. Sherman gives the carbohydrate content of cream meal as 79.0 per cent, the value for flask C 1, when calculated on the wet basis, was 78.3 per cent.

Table XVI

Sugar Content of Mashes Prepared by Hydrolyzing Various Starchy Materials with Various Concentrations of Acid.

Flask No.	Dextrose gms. per 100 ml.	Dextrose in filtrate (gms.)	Total dextrose (gms.)	Gms. dry starchy material	% dextrose dry starchy material
S 5	8.71	26.6	27.0	26.7	101.0
C 5	7.12	20.3	22.1	26.8	82.5
Y 5	7.08	20.2	21.9	26.7	82.0
S 1	8.63	26.2	26.8	26.7	100.4
C 1	7.55	22.3	23.4	26.8	87.3
Y 1	7.47	21.9	23.2	26.7	86.8
C 2	7.59	22.7	23.5	26.8	87.6
Y 2	7.40	22.2	22.9	26.7	85.8

The average conversion of corn starch was 97.5 per cent of theoretical. For all practical purposes 0.05 N sulfuric

acid gave the best conversion of corn starch, while 0.1 N acid gave the best conversion of the cereal meals. Higher concentrations of mash would probably require increased amounts of acid in order to bring about a good conversion.

Each flask was inoculated with 25 ml. of a 35-hr. inoculum of yeast No. 43 grown in the presence of a small amount of magnesium sulfite, and then 30 gms. of magnesium sulfite was added to each flask. Two different flasks of inoculum were used, and the glycerol carried over with the inoculum into the fermentation mashes makes a correction necessary. For series 5 and 2 the correction amounted to 0.08 gm. of glycerol, and for series 1 the correction amounted to 0.05 gm. of glycerol. A correction for the amount of dextrose carried over from the inoculum would be negligible since the inocula had stopped fermenting vigorously several hours before they were used. The experimental flasks were agitated at intervals, and at the end of four days of fermentation they were analyzed. The averaged results are given in Table XVII.

It may be concluded from these data that dry-milled corn as typified by the cream meal and yellow corn meal can serve as a suitable source of fermentable sugars when hydrolyzed by 0.1 N sulfuric acid. These meals would undoubtedly be somewhat cheaper than corn starch, but corn starch would require less acid and the problem of recovering the glycerol should be simpler from an acid-hydrolyzed corn starch mash.

Table XVII

Fermentation in the Presence of Magnesium Sulfite of Starchy Materials Hydrolyzed by Various Concentrations of Acid.

Flask No.	Glycerol found (gms.)	Corrected Wt. glycerol (gms.)	% Glycerol formed from Dextrose	% Glycerol formed from Dry starchy material
S 5	5.10	5.02	18.9	18.8
C 5	3.77	3.69	18.2	13.8
Y 5	3.68	3.60	17.8	13.5
S 1	5.10	5.05	19.3	18.9
C 1	4.51	4.46	20.0	16.6
Y 1	4.36	4.31	19.7	16.1
C 2	4.72	4.64	20.4	17.3
Y 2	4.64	4.56	20.5	17.1

6. The fermentation of various concentrations of acid-hydrolyzed starch

Duplicate samples of 30, 39, 51, and 60 gms. of corn starch were weighed into 500 ml. Erlenmeyer flasks, and then to each flask 300 ml. of 0.05 N sulfuric acid was added. The mashes were gelatinized and then cooked for four hours at 25 lbs. pressure. After hydrolysis samples were removed from each flask and analyzed for reducing sugar. The averages of the results are given in Table XVIII.

Table XVIII

Sugar Concentrations in Mashes Containing Various Amounts of Acid-Hydrolyzed Starch.

Flask No.	Gms. Starch	Gms. of dextrose equiv. to starch*	Amount of Dextrose found (gms.)	% Conversion
A	30	27.7	27.7	100.0
B	39	35.9	37.1	103.0
C	51	47.0	46.5	99.0
D	60	55.3	53.4	96.5

*Calculated from the value of 92.2% dextrose content obtained by A. O. A. C. acid-hydrolysis method for the analysis of starch

The conversions were all good although they decreased slightly at the higher starch concentrations. The values obtained were of course subject to the error that all reducing materials were calculated as dextrose which they might not be. One gram of calcium carbonate and one milliliter of steep water were added to each flask. Magnesium sulfite was added to each flask in amounts equal to the total weight of dextrose present. All flasks were inoculated with 30 ml. of a 27-hr. culture of yeast No. 43. When the flasks were analyzed on the fifth day, it was noticed that some of the flasks were still gassing strongly; so analyses were run again at two later dates. The results of these analyses are shown in Table XIX.

Table XIX

Fermentation of Various Concentrations of Acid-Hydrolyzed Starch in the Presence of Magnesium Sulfite.

Flask	Per cent glycerol on dextrose		
	5 days	7 days	21 days
A	17.1	19.3	18.3
B	14.3	17.8	18.1
C	11.1	15.0	19.5
D	8.6	12.1	18.6

The fermentations were completed more rapidly at lower sugar concentrations, but as the fermentations progressed the yields of glycerol all approached the same level. If the results obtained on the fifth day are extrapolated, by the method of Hickey (1941), back to zero concentration of sugar, we find the same limit of 25 per cent glycerol obtainable from dextrose as was obtained by Hickey. However, it is readily apparent from the data in Table XIX that this calculation is good only for short periods of fermentation and has no meaning when the fermentations are allowed to proceed to completion.

C. The Fermentation of Acid-Hydrolyzed Starch in the Presence of Sodium Sulfite

Considerable speculation was made as to why the yields of glycerol in the fermentations of dextrose in the presence of magnesium sulfite seldom exceeded 20-25 per cent of the weight of dextrose. It was noted that the total concentration of magnesium sulfite present in 5 ml. of the fermentation medium required on the average about 25 ml. of 0.1 N iodine solution and this would be equivalent to a concentration of 2.6 gms. of the sulfite per 100 ml. of medium. In turn this amount of magnesium sulfite would be equivalent to a concentration of about 3.1 gms. of sodium sulfite per 100 ml. Since the concentration of sugar present in the medium averaged about 10 per cent, the ratio of sodium sulfite (equivalent to magnesium sulfite) to sugar would be in the neighborhood of 30 per cent sulfite on sugar by weight. If one now looks at the yields of glycerol obtained by other investigators who have studied the effect of varying the ratio of sulfite to sugar, an interesting correlation may be seen. For instance, in the article by Gehle (1922) the following results were given:

% Sodium sulfite on sugar	% Glycerol formed from sugar
25	17.9
33	21-23
50	24-26
75	29-32
100	33
133	35

It was noted above that the amount of magnesium sulfite present in the fermentation medium was approximately equivalent to 30 per cent sodium sulfite on sugar. From Gehle's data it can be seen that such a ratio would correspond to a yield of about 20 per cent glycerol on sugar which value agrees remarkably well with many of the results obtained in the fermentations of dextrose in the presence of magnesium sulfite.

Since the amount of magnesium sulfite present in solution is limited by the solubility of this salt, it may be readily seen that the yields of glycerol obtained by fermentations in the presence of magnesium sulfite cannot be as high as when the more soluble sodium sulfite is used. Neuberg (1919) also noted this fact in his investigations on the use of the slightly soluble sulfites as acetaldehyde fixatives. Lowering the pH would increase the amount of magnesium sulfite (or bisulfite) going into solution, but unfortunately the toxicity of the bisulfite would also increase.

1. The fermentation of acid-hydrolyzed starch in the presence of varying amounts of sodium sulfite

a. Two hundred twenty-five grams of starch was suspended in 3 liters of 0.02 N sulfuric acid, gelatinized over a burner, and then cooked for four hours at 25 lbs. pressure. To the resulting hydrolyzate 15 gms. of calcium carbonate was added and the solution filtered. The sugar concentration of the hydrolyzate was 6.75 gms. per 100 ml. or a total of 204.5 gms. which is equivalent to a conversion of 98.5 per cent of theoretical. The recorder electrode assembly was immersed in the medium and 15 ml. of steep water and 72 gms. of sodium sulfite were added. The pH was adjusted to 8.0 and then 330 ml. of a 30-hr. culture of yeast No. 43 grown on a 10 per cent dextrose-steep water medium containing 7.2 gms. of sodium sulfite was added which brought the concentration of sodium sulfite to 2.34 gms. per 100 ml. or 35 per cent sulfite on sugar.

The pH fell from 7.8 to 6.8 in about 20 hours and remained at that level till the fermentation was sixty-six hours old and the fixed sulfite had reached a maximum value. At this point the pH began to increase rapidly and the fixed sulfite decreased slightly indicating that the fermentation was completed. Soon after the pH had begun rising signs of contamination became apparent. This was easily understandable because the medium had become alkaline and the free sulfite had fallen to a concentration requiring only 2.7 ml. of

iodine per 5 ml., and sulfite sufficient for a value of 5 ml. of iodine is usually the minimum amount required to inhibit the growth of contaminants even in slightly acid solutions.

A total of 49.0 gms. of glycerol was present in the medium at sixty-six hours. After all necessary corrections were made, this amounted to a yield of 21.0 per cent glycerol formed from the sugar. This yield checks rather well with the amounts obtained by Gehle and other workers and is very close to the average yield of glycerol obtained in the magnesium sulfite fermentations.

b. In this fermentation the per cent sulfite on sugar was increased to 43.5 per cent, other factors remaining constant. The same general procedure for hydrolyzing the starch was used as described above with the exception that 0.04 N sulfuric acid was used. The resulting hydrolyzate contained 6.68 gms. of dextrose per 100 ml. or a total of 207 gms. which amounted to a theoretical conversion of the starch. After placing the recorder electrode assembly in the medium, 90 gms. of sodium sulfite and 15 ml. of steep water were added, and the pH adjusted to 7.8. As an inoculum 350 ml. of a 41-hr. culture of yeast No. 43 containing initially 30 gms. of dextrose and 9.2 gms. of sodium sulfite was added. The final concentration of sodium sulfite amounted to 2.94 gms. per 100 ml. or 43.5 per cent on sugar.

In seventy hours the fixed sulfite reached a maximum

value equivalent to 17.3 ml. of 0.1 N iodine per 5 ml. of medium. However, the pH values did not follow the same trend as in the previous fermentation. The pH fell to 7.0 in twenty hours and remained at that level for about forty-eight hours more and then began increasing to 7.3. It stayed at 7.3 until the fermentation was seventy hours old and then increased rapidly to a value of 7.8. The total amount of glycerol present in the medium amounted to 55.0 gms. which gave a yield of 23.2 per cent glycerol on dextrose. This yield represented an increase of 2.2 per cent over the previous fermentation, and agreed with the values obtained by Gehle.

c. The medium for this fermentation was prepared in the same way as the medium described in part a of this experiment; the sugar concentration amounted to 6.6 gms. per 100 ml. or a total of 202.6 gms. for the whole medium before inoculation. To the medium were added 113 gms. of sodium sulfite, 15 ml. of steep water, and 330 ml. of a 45-hr. inoculum of yeast No. 43 containing originally 10 per cent dextrose and 13 gms. of sodium sulfite. These additions brought the concentration of sodium sulfite in the medium to 3.4 gms. per 100 ml. or 56 per cent sulfite on sugar. The pH fell to 7.0 in thirty hours and then to 6.8 in forty-five hours. After eighty hours the pH began increasing slowly reaching 7.5 in one hundred seventeen hours. The maximum value for fixed sulfite occurred at this time. The glycerol present in the medium amounted to a

total of 54.3 gms. or a yield of 23.4 per cent on original sugar. This yield was only slightly higher than that obtained in the previous fermentation, and the fermentation took considerably longer to go to completion.

It is evident from the data obtained in these three fermentations that for the lower concentrations of sodium sulfite the yields obtained from acid-hydrolyzed starch agreed rather well with the results obtained by other investigators who used dextrose or sucrose as the fermentable substrate. The agreement with the yields obtained by using magnesium sulfite as the aldehyde fixing agent were also good. However, in fermentation c the length of time required for the fermentation to cease, and the very slight increase in yield indicated that the yeast was not as active as in the lower sulfite concentrations. A few more experiments with still higher sulfite concentrations showed that the yeast definitely did not ferment the sugar under these conditions, since the yields of glycerol dropped to extremely low levels. Experiments described in a later section showed that when a fermented beer is used as an inoculum the yeast population is not high enough to carry on the complete fermentation of the sugar when the sulfite concentration exceeds 3 to 4 gms. per 100 ml. in sugar concentrations such as were used in the previous fermentations.

2. The fermentation of acid-hydrolyzed starch in 10-per cent sodium sulfite solution by large amounts of yeast

This experiment is included in this section to show that acid-hydrolyzed starch will ferment satisfactorily in high concentrations of sodium sulfite when a very large inoculum of yeast is used. One hundred thirty grams of starch was suspended in 1200 ml. of 0.03 N sulfuric acid, gelatinized, and then cooked for four hours at 25 lbs. steam pressure. The resulting hydrolyzate, containing 9.49 gms. of dextrose per 100 ml. (97 per cent conversion), was divided into four 300 ml. portions and to two of the portions calcium carbonate was added to neutralize the acid. To all four of the flasks 30 gms. of sodium sulfite was added followed by the addition of one-quarter of a yeast cake. The pH of the flasks containing calcium carbonate was 8.0; the pH of the other two flasks was 7.5.

When the flasks were analyzed on the fourth day the two flasks containing calcium carbonate showed slightly higher yields of glycerol than the other two flasks, but on the fifth day all flasks showed the same amount of glycerol. The per cent glycerol formed from the sugar amounted to 28.8 per cent. It is quite evident that acid-hydrolyzed starch serves as well as purified dextrose for a fermentable substrate in the presence of sodium sulfite.

D. The Effect of Varying the Strain of Yeast on the Yield of Glycerol Obtained from Various Sugars

1. The fermentation of dextrose in the presence of magnesium sulfite by various strains of yeasts.

The four strains of yeasts used were as follows: three Saccharomyces cerevisiae strains designated as No. 43, No. 16, and No. 51, and also a strain of S. ellipsoideus var. Steinberg designated as No. 53. These cultures were carried through three successive 36-hour sub-cultures in a 5-per cent dextrose-steep water medium starting from a slant. The third transfer was used as the inoculum. For the fermentation media eight flasks each containing 200 ml. of a 10-per cent dextrose-steep water solution and 20 gms. of magnesium sulfite were used. To duplicate flasks 20 ml. of inoculum of the desired yeast strain was added. The flasks were shaken periodically and were analyzed at the end of the fifth day of fermentation. The average results of the analyses are shown in Table XX.

Table XX

Effect of Various Strains of Yeasts on the Fermentation of Dextrose in the Presence of Magnesium Sulfite.

Yeast No.	Gms. Glycerol	Gms. Dextrose	% Glycerol on dextrose
53	3.58	20	17.9
43	3.92	20	19.6
16	3.28	20	16.4
51	3.74	20	18.7

From these data it is apparent that yeast No. 43 gave the highest yield of glycerol, but the differences between the three strains of S. cerevisiae giving highest yields of glycerol were not great.

2. The fermentation of maltose in the presence of magnesium sulfite by various strains of yeasts

The same strains of yeasts and the same general procedures were used as were described in the previous experiment. Instead of dextrose technical grade maltose was used as the fermentable substrate, however. When the fermentations were five days old they were analyzed and the averaged results are shown in Table XXI.

Table XXI

Effect of Various Strains of Yeasts on the Fermentation of Maltose in the Presence of Magnesium Sulfite.

Yeast No.	Gms. Glycerol	Gms. Maltose*	% Glycerol on maltose
16	2.23	17.6	12.7
43	0.91	17.6	5.2
51	2.15	17.6	12.2
53	2.43	17.6	13.8

*Twenty grams of tech. maltose is equivalent to 17.6 gms. of maltose hydrate.

Strain No. 43, although the best for the fermentation of dextrose, was by far the poorest for the fermentation of maltose. Saccharomyces ellipsoideus, No. 53, was the best of

the four strains of yeast tried for the fermentation of maltose. The yields of glycerol obtained from maltose were noticeably less than those obtained from dextrose.

3. The fermentation of various sugars in the presence of magnesium sulfite

In this series 20 gms. each of C. P. maltose hydrate, lactose hydrate, levulose, C. P. sucrose, and C. P. dextrose were weighed into 300-ml. Erlenmeyer flasks in duplicate. To each flask 200 ml. of tap water and one milliliter of steep water were added, and the media were sterilized for fifteen minutes at 15 pounds' pressure. After the flasks had cooled, 25 gms. of magnesium sulfite and 20 ml. of a 42-hr. culture of yeast No. 43 grown on a 5 per cent dextrose-steep water medium were added. At the end of the fifth day of fermentation the dextrose, sucrose, and levulose media had almost ceased to show signs of gassing, while the lactose and maltose media still had shown no signs of activity. The averaged results of the analyses are shown in Table XXII.

Lactose is known not to be fermented by many strains of yeast; so the low yield of glycerol from lactose is understandable, but the poor fermentation of maltose is still without explanation. Levulose, sucrose, and dextrose all fermented satisfactorily. The apparently lower yield of glycerol obtained from levulose may be caused by the fact that the sample of levulose used showed signs of containing considerable moisture.

Table XXII

Yields of Glycerol Obtained from Various Sugars Fermented in the Presence of Magnesium Sulfite.

Sugar	Gms. Glycerol	Gms. Sugar	% Glycerol on sugar
Lactose	0.30	20	1.5
Maltose	0.32	20	1.6
Sucrose	3.64	20	18.2
Dextrose	3.68	20	18.4
Levulose	3.10	20	15.5

4. The fermentation of various sugars in the presence of sodium sulfite

This series was run after much of the work on the fermentation of dextrose in the presence of sodium sulfite by large amounts of yeast, described in a later section, had been completed. It is included here because of the similarity to the previous series. Small volumes were employed since one of the sugars used, namely trehalose, is a rare sugar and quite expensive.

Into four separate test tubes of about 15-ml. capacity one gram of sodium sulfite, one drop of steep water, 10 ml. of tap water, and one gram of sugar were placed. Each tube was then inoculated with a small piece of a yeast cake which actually represented a very large inoculum. Four days later the contents of the tubes were analyzed; the results are shown in Table XXIII.

Table XXIII

Yields of Glycerol Obtained from Various Sugars Fermented in the Presence of Sodium Sulfite.

Sugar*	Total gms. glycerol	% Glycerol on sugar
Trehalose	0.002	0.2
Maltose	0.010	1.0
Sucrose	0.300	30.0
Dextrose	0.290	29.0

* All sugars were of the C. P. grade. The trehalose and maltose were monohydrates.

The yields of glycerol from both sucrose and dextrose were excellent while the yields from the other two sugars were negligible. Actually the yield of glycerol obtained from sucrose when calculated on the basis of the hexose equivalent of sucrose amounted to 28.8 per cent, almost identical with the yield from dextrose.

E. The Fermentation of Dextrose in the Presence of Sulfite by Large Amounts of Yeast

In experiments described in previous sections certain of the results obtained seemed to indicate that not only the ratio of sulfite to sugar but also the actual concentration of sulfite seemed to influence the yield of glycerol. Other results pointed to the fact that the size of the inoculum was an important factor. In order to simplify procedures and to dispense with as many unwanted variables as possible, dextrose

was used as the fermentable substrate, while sodium sulfite was used in almost all of the fermentations as the aldehyde binding agent. So as to insure the presence of enough yeast the use of yeast cakes was resorted to. Much of the work reported in the literature involves the use of such large masses of yeast.

The use of large amounts of yeast, in the form of pressed yeast, as inocula is ordinarily not practical on a commercial scale. High yeast concentrations could be obtained by aerating a sugar-containing medium, and after the yeast had multiplied sufficiently the sulfite and, perhaps, more sugar could be then added. Such a procedure has been patented by Hildebrandt and Erb (1939).

1. The effect of nutrients on the fermentation of dextrose in the presence of sodium sulfite

Certain indications from previous fermentations and from reports found in the literature seemed to point to the fact that the yeast either does not multiply in the presence of high concentrations of sulfite or else only multiplies to a very small degree. If this is true, the yeast should not require as much nutrient material as in a normal alcoholic fermentation. For the nutrient medium a 5-per cent dextrose solution was used containing salts and yeast extract in the amounts described for the optimum semi-synthetic medium mentioned in an earlier part of this thesis. For control

purposes a solution of 5-per cent dextrose in tap water was employed. The volume of the medium used in each case was 300 ml., and sodium sulfite in the amounts indicated in Table XXIV was added. Each flask was inoculated with one-half of a yeast cake (about 5 gms. of yeast), and the series was analyzed five days after inoculation. The results are shown in Table XXIV.

Table XXIV

Effect of Nutrients on the Yield of Glycerol.

% Sulfite on sugar	Added nutrients	% Glycerol on sugar
50	present	20.2
50	absent	20.6
100	present	26.2
100	absent	23.9
150	present	26.9
150	absent	25.4

As can be seen from these data, the presence of nutrients apparently does increase the yield of glycerol. In later work the synthetic medium was not used since steep water in conjunction with tap water was found to be as satisfactory and is certainly more convenient and cheaper.

2. The fermentation of various concentrations of dextrose in a 10-per cent sodium sulfite solution

The media for this series were prepared by distributing 2800 ml. of a 10-per cent sodium sulfite solution containing

12 ml. of steep water in 14 portions of 200 ml. in 300-ml. Erlenmeyer flasks. To duplicate flasks increasing amounts of dextrose were added as indicated in the following table, and each flask was inoculated with one-quarter of a yeast cake. After three days of fermentation the flasks were analyzed and the averaged results are shown in Table XXV.

Table XXV

Effect of Varying Dextrose Concentrations on the Yield of Glycerol in a 10-Per Cent Sodium Sulfite Solution.

Gms. dextrose	Per cent dextrose	% Sulfite on dextrose	Gms. glycerol	% Glycerol on dextrose
6	3.0	333	1.39	23.1
10	5.0	200	2.70	27.0
15	7.5	133	4.38	29.3
20	10.0	100	6.00	30.0
25	12.5	80	7.38	29.6
30	15.0	67	8.67	28.9
40	20.0	50	9.23	23.1

Actually the percentages of sulfite and dextrose in the media were slightly less than the values shown for the flasks containing the higher concentrations of sugar because the addition of the sugar increased the volume slightly. The highest yield of glycerol occurred at a concentration of 10 per cent dextrose and the yields dropped as the sugar concentration increased above 10 per cent. Precautions must be observed in interpreting the data obtained from a series prepared in this manner because not only does the sugar

concentration vary, but also the ratio of sulfite to sugar varies.

It had been noticed in a previous fermentation that the yield of glycerol increased after longer periods of fermentation when higher sugar concentrations were used, but analyses on the series just described after five days had elapsed showed only negligible gains. Another series almost exactly duplicating this series was run at a later date. The only differences were that the fermentations were run in a volume of 300 ml. and were allowed to ferment for four days before being analyzed. The yields followed the same trend but tended to be one to 2 per cent lower and the drop after the maximum at 10 per cent dextrose was not as marked.

If the concentration of glycerol is plotted against the concentration of dextrose, an almost linear relation from zero to 12 per cent dextrose is obtained.

3. The fermentation of various concentrations of dextrose in the presence of varying amounts of sodium sulfite

The following experiment represents a combination of several series carried out at different times, but combined here because of the similarity of purpose and preparation. The media for each series were prepared by dissolving the required amount of sugar and steep liquor in a given volume of tap water and then distributing in 200-ml. portions between 300-ml. Erlenmeyer flasks containing previously weighed amounts

of sodium sulfite. All media, fermented in duplicate, were inoculated with one-quarter of a yeast cake. The fermentations were incubated at 30° C. and the flasks were shaken occasionally to keep the yeast in suspension. The 5- and 10-per cent dextrose series were allowed to ferment three days, the 7.5-per cent dextrose series fermented four days, and the 12.5-per cent dextrose series fermented five days.

In Table XXVI the averaged results for the various duplicates are given, and in Figures 1 and 2 some of the data are plotted. Actually the concentrations of sulfite and dextrose shown in the table are subject to a small variation because of the change in volume when the sulfite was dissolved in the medium, but the error is small enough to be disregarded for all practical purposes.

The maximum yield of glycerol occurred at a dextrose concentration of 10 per cent and a sulfite concentration of 10 per cent. The highest amount of glycerol present in the medium was found in the flasks containing 12.5 per cent dextrose and 15.6 per cent sodium sulfite, however. It is difficult to determine from these data whether the ratio of sulfite to dextrose or the actual concentration of sulfite is the more important factor in determining the yield of glycerol. Undoubtedly the two factors are both involved. Why the yields of glycerol should drop in the higher concentrations of sulfite for the 10- and 12.5-per cent dextrose series is another questionable point. The latter phenomenon,

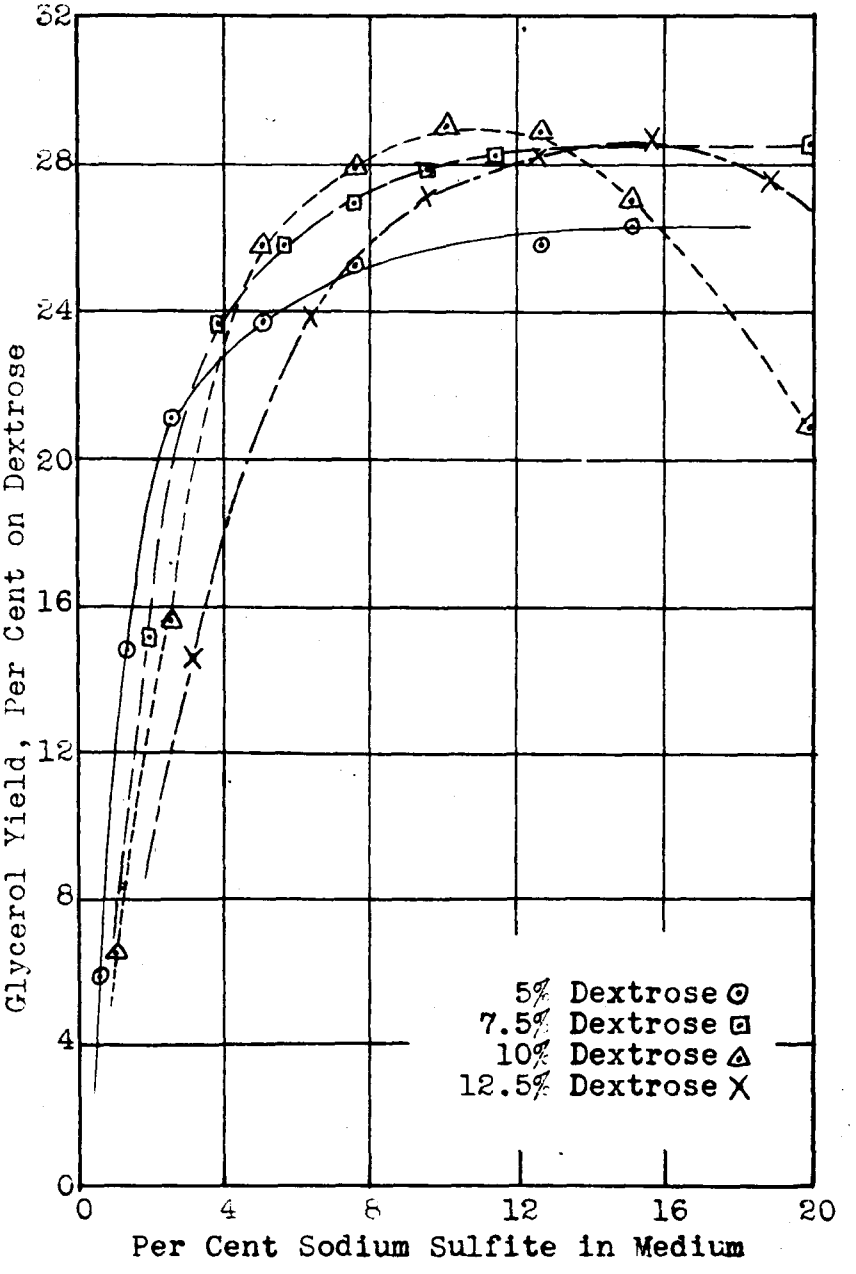
Table XXVI

Yields of Glycerol Obtained by Fermenting Various Concentrations of Dextrose in the Presence of Varying Concentrations of Sodium Sulfite Using Large Inocula.

% Dextrose in medium	% Sulfite in medium	% Sulfite on dextrose	Gms. Glycerol per 100 ml.	% Glycerol on dextrose
5	0.5	10	0.29	5.9
"	1.3	25	0.74	14.8
"	2.5	50	1.05	21.1
"	5.0	100	1.19	23.8
"	7.5	150	1.26	25.2
"	10.0	200	(1.24)	(24.7)
"	12.5	250	1.29	25.8
"	15.0	300	1.32	26.3
"	20.0**	400	1.42	28.8
7.5	1.9	25	1.13	15.1
"	3.8	50	1.78	23.7
"	5.6	75	1.94	25.8
"	7.5	100	2.01	26.9
"	9.4	125	2.09	27.9
"	11.3	150	2.11	28.2
"	20.0**	267	2.14	28.5
10	1.0	10	0.61	6.4
"	2.5	25	1.56	15.6
"	5.0	50	2.57	25.8
"	7.5	75	2.79	27.9
"	10.0	100	2.89	29.0
"	12.5	125	2.88	28.9
"	15.0	150	2.70	27.0
"	20.0	200	2.09	20.9
"	20.0*	200	2.62	26.2
12.5	3.1	25	1.82	14.6
"	6.3	50	2.98	23.9
"	9.4	75	3.38	27.1
"	12.5	100	3.53	28.2
"	15.6	125	3.59	28.7
"	18.8	150	3.45	27.6

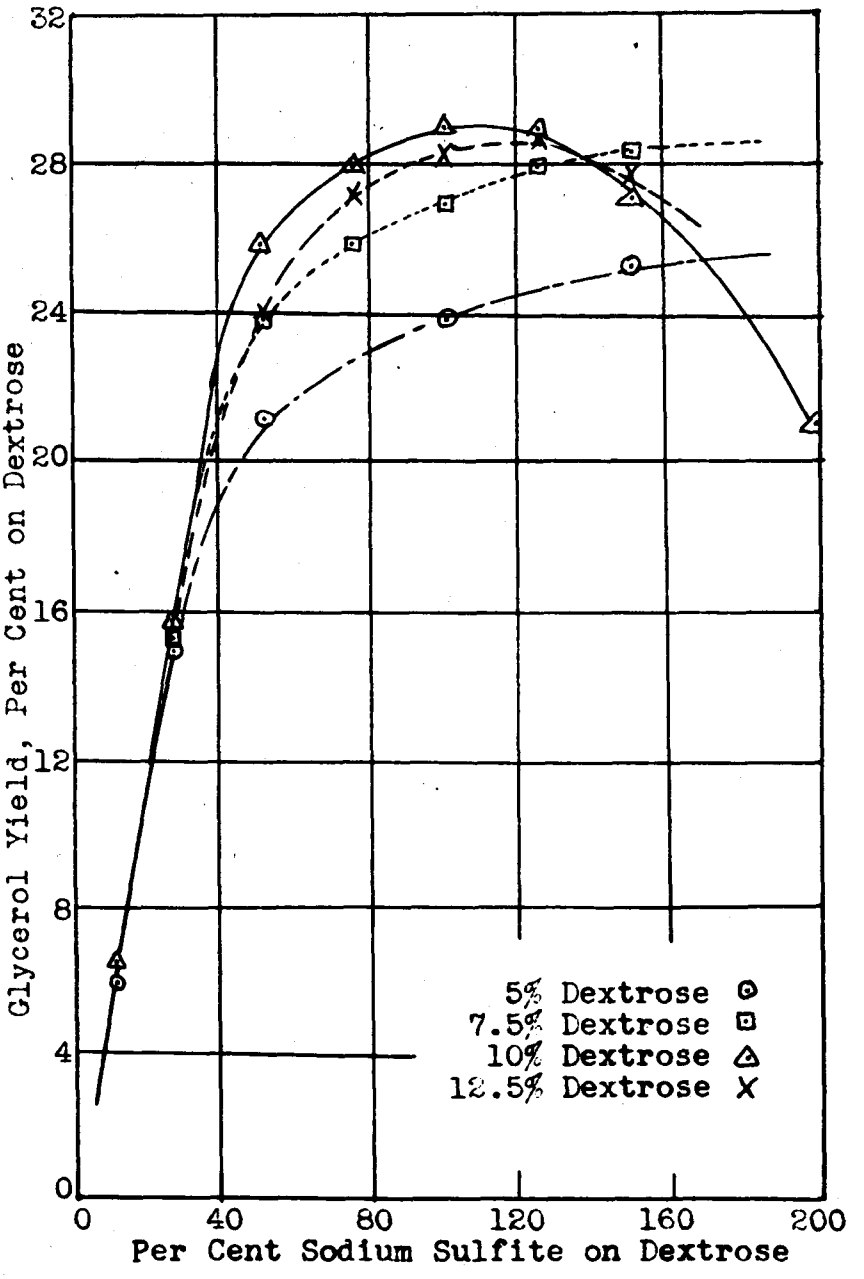
* 8 days old

** 300 ml. volume



The Effect of Varying the Concentration of Sodium Sulfite on the Yields of Glycerol.

Figure 1.



The Effect of Varying the Ratio of Sodium Sulfite to Dextrose on the Yields of Glycerol.

Figure 2.

in all likelihood, would disappear if the fermentations were allowed to proceed for a longer period of time. It is also to be noted that the yields of glycerol for the same concentration of sulfite decrease with decreasing concentration of dextrose. In general it appears that a 10-per cent dextrose solution containing 10 per cent sodium sulfite furnishes the highest yields of glycerol.

4. A pH recording of the fermentation of a 10-per cent dextrose, 10-per cent sodium sulfite solution

Exactly 3 liters of a solution containing 300 gms. of dextrose, 300 gms. of sodium sulfite and 6 ml. of steep liquor in tap water was prepared and the recorder electrode assembly was immersed in this medium. As an inoculum two yeast cakes were used. Samples were removed at intervals and the results of the analyses are shown in Table XXVII.

Table XXVII

pH Recording of the Fermentation of a 10-Per Cent Dextrose, 10-Per Cent Sodium Sulfite Solution.

Age (hrs.)	pH	Ml. of 0.1 N Iodine equiv. to fixed sulfite
1	9.0	1.6
4	8.5	2.2
22	7.0	13.8
35	6.9	21.2
46	7.0	26.2
58	7.0	29.1
70	7.0	30.0
94	7.0	30.1

The maximum yield of glycerol occurred at seventy hours, although the fermentation may not have actually been completed, since the pH was not rising and the fixed sulfite was still increasing very slowly. The yield of glycerol amounted to 27.6 per cent on dextrose. It seems that the yeast tends to adjust the acidity of the medium so that the sulfite toxicity is not too great, and yet the acidity is high enough to enable the cells to function as nearly normally as possible.

5. The effect of temperature, surface-volume ratio, and age of yeast cake on the yields of glycerol

a. In many reports in the literature conflicts are to be found as to what the optimum temperature is for the production of glycerol by fermentation. Some investigators claim that 30° C. is the best temperature, while others claim that higher yields are obtained by incubating the mashes at 37° C. Of course it may be possible that the discrepancies are due to the use of different yeasts or to some other factor. In order to see if temperature changes would affect the yields of glycerol under the conditions of fermentation described heretofore, the following fermentations were prepared.

A solution containing 80 gms. of dextrose, 80 gms. of sodium sulfite, and 4 ml. of steep water in 800 ml. of tap water was distributed in four equal portions between separate 300-ml. Erlenmeyer flasks. Each flask was inoculated with one-quarter of a yeast cake, and two flasks were placed in an

incubator at 30° C. while the other two flasks were placed in an incubator at 37° C. After three and one-half days of fermentation, the fermentations incubated at the lower temperature showed a yield of 28.9 per glycerol on dextrose, while those incubated at the higher temperature showed a yield of 29.5 per cent glycerol on dextrose. Therefore, by increasing the temperature of incubation 7° C. the yield of glycerol was raised by only 0.6 per cent, which is not a really significant change.

b. Previously it had been noted that fermentations carried out in small volumes (25 ml. or thereabouts) did not give as high yields of glycerol as might be expected. It was believed that in all probability this phenomenon was associated not with the small volume but rather with the surface-volume ratio (S/V). The ratio is obtained by dividing the area of the surface of the medium ($cm.^2$) by the volume of the medium ($cm.^3$).

A total of 1300 ml. of a solution containing 130 gms. of sodium sulfite, 130 gms. of dextrose, 6 ml. of steep water, and one yeast cake was prepared and distributed between five Erlenmeyer flasks of different sizes in the proportions indicated in Table XXVIII. When the flasks were four days old the contents were analyzed and the results of the analyses are shown in the table.

Table XXVIII

Effect of the Surface-Volume Ratio on the Yields of Glycerol.

Vol. of medium (ml.)	Size of flask (ml.)	S/V Ratio	% Glycerol on dextrose
10	50	1.26	18.5
50	125	0.57	26.8
200	300	0.17	29.1
300	500	0.15	29.2
700	1000	0.11	29.2

The decrease in the yield of glycerol when the S/V ratio becomes greater than 0.2 may be caused by the effect of the oxygen of the air on the metabolism of the yeast, or perhaps it may only be an apparent decrease brought about by the loss of acetaldehyde either through volatilization or oxidation which would decrease the amount of fixed sulfite in the medium. In any case the S/V ratio should be less than 0.2 to cancel out this decrease in the yield of glycerol whether real or apparent.

In a previous experiment (Section D, experiment 4) volumes of 10.5 ml. of medium were fermented in test tubes, the S/V ratio being 0.12. The yield of glycerol from dextrose amounted to 29.0 per cent, thus confirming the fact that the S/V ratio and not the volume is the critical factor.

c. Since the yeast cakes used for massive inoculations were known not to be of the same age when used, it was

thought worthwhile to see if the age of the yeast cake had any appreciable effect on the yield of glycerol. A solution containing 10 per cent dextrose, 10 per cent sodium sulfite, and added steep water was divided in 300-ml. portions between four 500-ml. Erlenmeyer flasks. The media in two of the flasks were inoculated with one-quarter of a yeast cake recently obtained while the media in the other two flasks were inoculated with the same amount of yeast from a cake that had been kept in a refrigerator for two weeks. The analyses of these flasks after three days of fermentation showed that the yields of glycerol were almost identical (28.2 per cent glycerol on dextrose); so it may be concluded that the age of the yeast cake, within limits, is not critical. Of course this is assuming that the yeast has been kept under refrigeration and not been allowed to deteriorate.

6. The fermentation of dextrose in the presence of magnesium sulfite

Since higher yields of glycerol were obtained from fermentation media containing high concentrations of sodium sulfite when large amounts of yeast were used as inocula, it was thought worthwhile to see what effect large inocula would have on the fermentation of dextrose in the presence of magnesium sulfite. To a solution of 30 gms. of dextrose in 600 ml. of tap water one yeast cake and 30 gms. of magnesium sulfite were added. The medium was continuously agitated by

an air-driven stirrer. In twenty hours the glycerol yield amounted to 19.9 per cent of the dextrose, and the fermentation was completed in that time. Although the fermentation took less time than similar fermentations employing smaller inocula the yield was not appreciably different.

A solution of 70 gms. of dextrose in 740 ml. of tap water containing one-third of a yeast cake, one gram of yeast extract, and 70 gms. of magnesium sulfite was fermented as in the previous experiment. At the end of twenty-three hours the yield of glycerol amounted to only 15.9 per cent of the initial dextrose, but in fifty-six hours the yield had reached a maximum of 19.8 per cent. This fermentation took longer to reach completion than the previous one, which might be expected, however, since the inoculum was smaller and the dextrose concentration higher. The presence of nutrient material did not seem to affect the yield of glycerol. It appears from these two fermentations that the use of large amounts of yeast does not increase the yields of glycerol when dextrose is fermented in the presence of magnesium sulfite.

F. The Effect of Varying the Amount of Inoculum on the Yields of Glycerol Obtained by Fermenting Dextrose in the Presence of Sodium Sulfite

Observations from previous fermentations indicated that ordinary inocula are not satisfactory for the fermentation of dextrose when high concentrations of sulfite are present. Such ordinary inocula are prepared by inoculating a fermentable medium, such as beer wort, with a strain of yeast, allowing the yeast to reach maximum growth, and then using the fermented beer for inoculation purposes. On the other hand dextrose in the presence of high concentrations of sulfite could be fermented readily by adding large amounts of pressed yeast. When an ordinary inoculum in the proportion of 10 per cent by volume is used to inoculate a fermentation medium, the yeast count in the inoculated medium is seldom greater than 30 (i.e. 30 x 250,000 yeast cells per ml.). The yield of glycerol obtained from the fermentation of a 10-per cent dextrose solution in the presence of 10 per cent sodium sulfite was only 2.6 per cent when such an inoculum was used. When large amounts of yeast were used an identical medium gave a yield of 29.0 per cent glycerol on dextrose. It is quite apparent therefore that the amount of yeast used does influence the yield of glycerol, and in the following experiments an attempt was made to see what relation existed between these various factors.

1. The fermentation of a 10-per cent dextrose, 10-per cent sodium sulfite solution by various amounts of yeast

Two liters of a solution containing 200 gms. of dextrose, 200 gms. of sodium sulfite, and 8 ml. of steep water was distributed evenly between ten 300-ml. Erlenmeyer flasks. To duplicate flasks 0.01, 0.05, 0.1, 0.25, and 0.5 of a yeast cake were added. The smaller fractions were obtained by suspending one-half of a yeast cake in distilled water, making a total volume of 50 ml., and then removing aliquot portions by means of a pipette. After four days of fermentation the fermentations were analyzed and the averaged results are shown in Table XXIX.

Table XXIX

Effect of Varying the Inoculum on the Yields of Glycerol Obtained by Fermenting a 10-Per Cent Dextrose, 10-Per Cent Sodium Sulfite Solution.

Fraction of yeast cake	Approx. initial yeast count*	% Glycerol on dextrose
0.01	36	5.3
0.05	185	28.8
0.10	350	29.1
0.25	920	30.1
0.50	1700	30.4

*These counts should be multiplied by 250,000 to obtain the actual number of cells per ml.

As can be seen from the data in the table, the yields of

glycerol for these concentrations of dextrose and sodium sulfite did not increase greatly when the yeast count was above about 200. The fermentations described in the previous sections wherein a quarter of a yeast cake was used as inoculum contained sufficient yeast to carry out the satisfactory conversion of dextrose to glycerol. On the other hand, the poor yield obtained in this experiment with a yeast count of 36 showed that a count of 30, usually obtained when an ordinary inoculum is employed, would definitely not be sufficient to bring about satisfactory fermentation of dextrose in solutions of high sulfite concentrations.

2. The fermentation of a 10-per cent dextrose solution in the presence of varying amounts of sodium sulfite by varying amounts of yeast

In the following series the fermentation medium was a 10-per cent dextrose solution containing 4 gms. of steep water per liter of solution. To the solution to be fermented enough yeast was added to bring the count to the desired level, and then the medium was distributed in 200-ml. portions between flasks which contained previously weighed out quantities of sodium sulfite. All fermentations were run in duplicate, and since the yeast was added to the whole batch of medium before splitting it up, the yeast counts in all flasks were practically identical. The fermentations were allowed to proceed for four days and then were analyzed. The combined

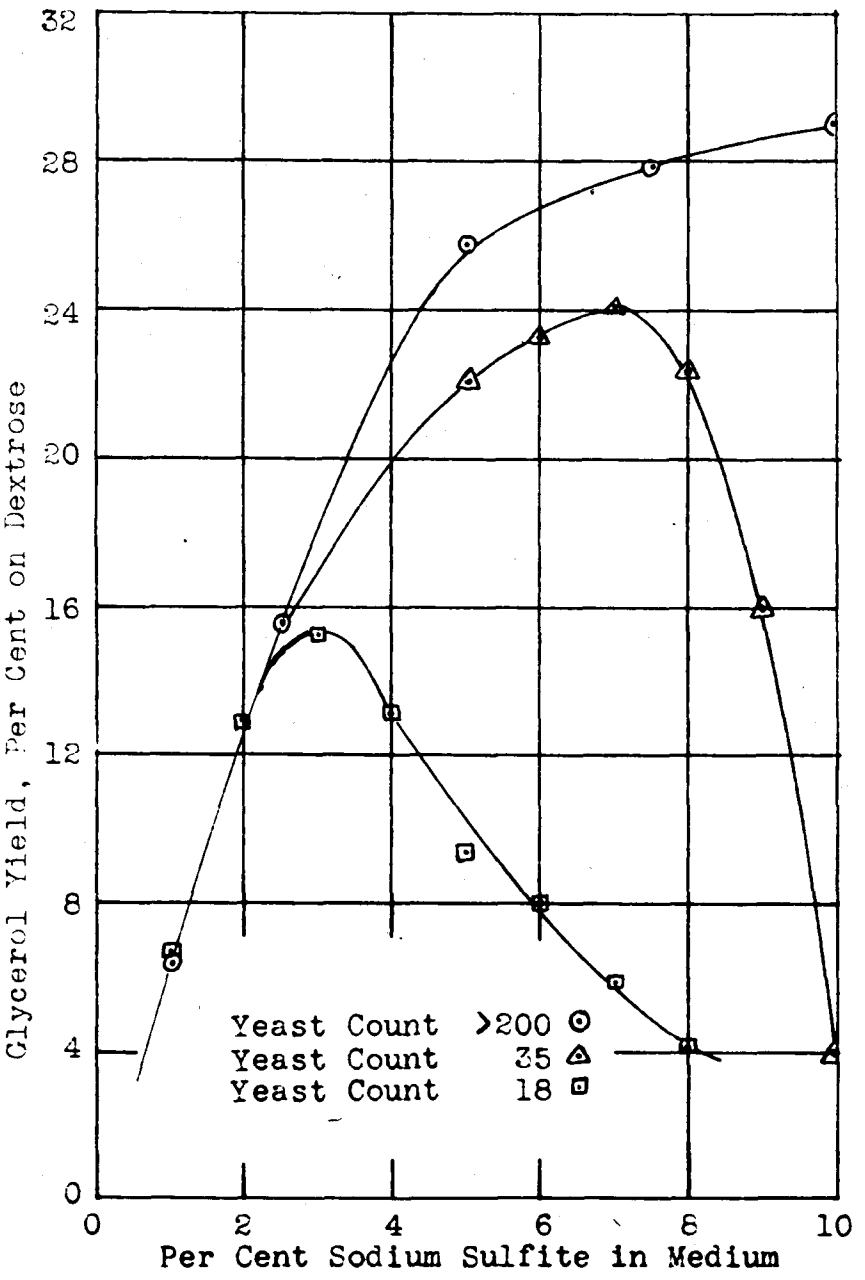
results of these series are shown in Table XXX. In Figure 3 these results and some data from Table XXVI are shown in graphic form.

Table XXX

Effect of Varying the Inoculum on the Yields of Glycerol from the Fermentation of a 10-Per Cent Dextrose Solution in the Presence of a Variable Amount of Sodium Sulfite

% Sulfite in medium	Initial yeast count	Final yeast count	% Glycerol on dextrose
1	18	-	6.7
2	"	-	12.9
3	"	-	15.2
4	"	-	13.1
5	"	-	9.3
6	"	-	8.0
7	"	-	5.9
8	"	-	4.1
1	35	100	6.5
5	"	81	22.1
6	"	62	23.3
7	"	64	24.1
8	"	50	22.4
9	"	36	16.0
10	"	(27)	4.0

It can be readily seen from the above data that a maximum yield of glycerol was obtained at 3-per cent sulfite concentration when the initial count was 18, and when the initial count was 35 the maximum occurred at 7-per cent sulfite concentration. From previous data a maximum was obtained at



The Effect of Varying the Initial Yeast Concentration on the Fermentation of a Ten Per Cent Dextrose Solution in the Presence of Varying Amounts of Sodium Sulfite.

Figure 3.

10-per cent sulfite concentration when the initial count was above 200. Therefore we may conclude that with increasing amounts of yeast the optimum amount of sulfite increases also, a limit being reached at a concentration of 10 per cent sodium sulfite. These data apply only to a 10-per cent dextrose solution. The following experiment indicated that for 5-per cent dextrose solutions the optimal amounts of sodium sulfite differ.

3. The fermentation of a 5-per cent dextrose solution by varying amounts of yeast in the presence of varying amounts of sodium sulfite

The following fermentations were prepared in a similar manner to those in the preceding experiment, the only difference being that a 5-per cent dextrose solution was used instead of a 10-per cent solution. The analyses after four days of fermentation are shown in Table XXXI.

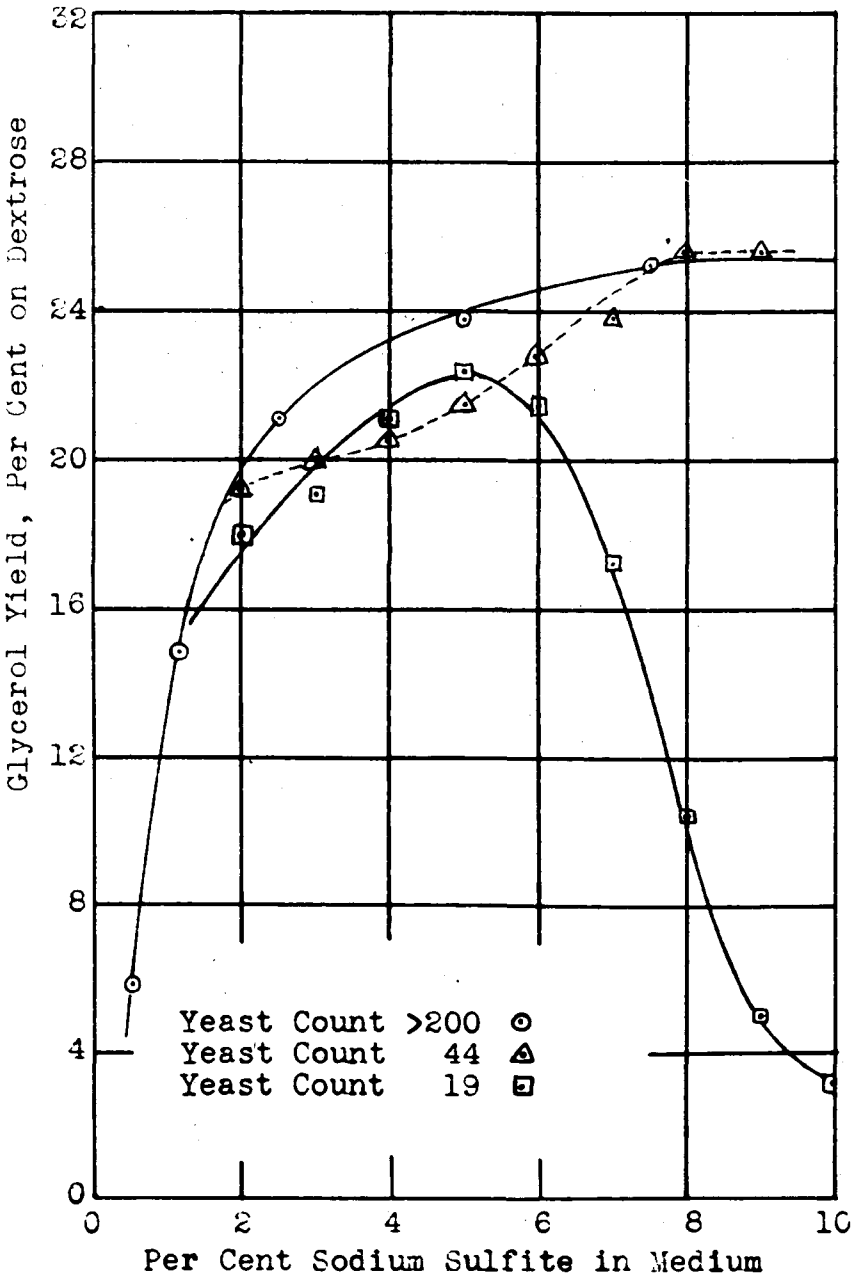
The maximum yield of glycerol occurred at 5 per cent sulfite when the initial count was 19, but when the initial yeast count was 44, the glycerol yield did not show a maximum but rather tended to level off above 8-per cent sulfite concentration. In previous fermentations where the yeast count was above 200 the yields of glycerol seemed to reach a maximum of 26.0 per cent at concentrations of sodium sulfite in the neighborhood of 10 per cent. In Figure 4 the data from Table XXI and some from Table XXVI are shown in graphic form.

Table XXXI

Effect of Varying the Inoculum on the Yields of Glycerol Obtained by Fermenting a 5-Per Cent Dextrose Solution in the Presence of Varying Amounts of Sodium Sulfite.

% Sulfite	Initial yeast count	% Glycerol on dextrose
2	19	18.0
3	"	19.1
4	"	21.1
5	"	22.4
6	"	21.5
7	"	17.2
8	"	10.4
9	"	5.0
10	"	3.2
2	44	19.2
3	"	20.0
4	"	20.5
5	"	21.5
6	"	22.8
7	"	23.9
8	"	25.6
9	"	25.6

One might assume that a given amount of yeast will only ferment a certain amount of dextrose at a given sulfite concentration. This assumption might explain some of the experimental data, but certainly not all of it. One interesting fact does stand out, however, namely that the maximum yields of glycerol are found at higher levels of sulfite concentration for a 5-per cent dextrose solution than for a 10-per cent dextrose solution when minimal amounts of yeast are used. It may also be



The Effect of Varying the Initial Yeast Concentration on the Fermentation of a Five Per Cent Dextrose Solution in the Presence of Varying Amounts of Sodium Sulfite.

Figure 4.

said that for a given concentration of sodium sulfite more yeast is required to ferment a 10-per cent dextrose solution than a 5-per cent solution.

4. The use of a regular inoculum for the fermentation of dextrose in the presence of sodium sulfite

The inoculum used in this series was a 42-hr. culture of yeast No. 43 grown on a 2.5-per cent dextrose-steep water medium. This inoculum might have been somewhat past the point of optimum activity of the yeast, but by using an inoculum of this age the correction for the amount of sugar carried over into the fermentation media was reduced to a negligible factor. In order to maintain the dextrose and sulfite concentrations at the proper levels a procedure was resorted to which brought the amount of the inoculum to about 11 per cent of the volume of the medium being inoculated. For instance, the 5-per cent dextrose flasks were prepared by dissolving 60 gms. of dextrose and 5 ml. of steep water in a total volume of 1080 ml., and then adding 120 ml. of inoculum, bringing the total volume of the medium to 1200 ml. Then this medium was divided between duplicate flasks containing 6, 10, and 14 gms. of sodium sulfite, each flask receiving 200 ml. of the medium. The 10-per cent dextrose flasks were prepared in a similar manner.

Initial counts of yeast in the various flasks varied from 15 to 19, but this is within the counting error. The

analyses were made after three days of fermentation, results being shown in Table XXXII.

Table XXXII

Effect of Using a Regular Inoculum on the Yields of Glycerol from Dextrose Fermented in the Presence of Varying Amounts of Sodium Sulfite.

% Sulfite in medium	% Dextrose in medium	% Glycerol on dextrose
3	5	21.8
5	"	22.8
7	"	21.8
1	10	6.6
2	"	12.7
3	"	19.7
5	"	17.9
7	"	15.3
9	"	6.9

As can be seen the maximum yield of glycerol for the 5-per cent dextrose media occurred at 5 per cent sulfite, and both the yield of glycerol and the concentration of sulfite agreed very well with previous results. The maximum for the 10-per cent dextrose fermentations occurred at 3 per cent sulfite, but, although the maximum was the same, the actual yield of glycerol was somewhat higher than the previous results obtained when small inocula were used.

5. The effect of the gradual addition of sulfite on the yield of glycerol

It has been reported in the literature, especially in patents, that the gradual addition of sulfite leads to higher yields of glycerol. One explanation of this effect could be that the yeast has a chance to multiply before the sulfite concentration reaches inhibitory levels. Since the bisulfite-aldehyde complex is not supposed to be toxic to yeast, the addition of sulfite in gradual amounts would keep the concentration of free sulfite or bisulfite at a low enough level so as not to be toxic to the yeast. In order to get an approximate idea as to the effect of the gradual addition of sulfite the following media were prepared.

Four flasks containing 300 ml. each of a 9-per cent dextrose-steep water medium were prepared and then each flask was inoculated to a count of about 40 with a suspension of a yeast cake in water. To each of two of the flasks 30 gms. of sodium sulfite was added at one time. To each of the other two flasks a total of 30 gms. of sodium sulfite was added in small amounts over a period of two days. At the end of four days of fermentation the media were analyzed. In those flasks where the sulfite had been added at one time the average yield of glycerol was 17.3 per cent, while in the other flasks the yields averaged 24.7 per cent. The final yeast counts were 38 and 80, respectively. Although the yields in the flasks to which all the sulfite had been added at one time were

higher than might be expected they still were 7 per cent lower than the yield found in the flasks to which the sulfite had been added gradually. The fact that the yeast count doubled over the initial count when the sulfite was added gradually indicated that the yeast grew to some extent in the initial stages of the fermentation. The yeast did not grow in the flasks to which all of the sulfite had been added at one time. It can be definitely said that the gradual addition of sulfite increases the yield of glycerol when the initial yeast count is below 200.

G. The Fermentation of Maltose in the Presence of Sodium Sulfite

It had been noticed in previous fermentations where enzyme-saccharified starchy materials were fermented in the presence of sulfites that poor yields, in comparison with dextrose fermentations, were always obtained. In fact, the highest yields averaged about 12 per cent of the maltose hydrate present at the beginning of the fermentation. Since maltose could be readily fermented in the absence of sulfite, as is evidenced by final sugar concentrations in the order of 0.1 per cent, the difficulty must arise because of the presence of sulfite. This was confirmed by previous work, and by the fact that a yeast culture, instead of becoming acclimatized to sulfite, failed to reproduce when carried on a maltose medium containing magnesium sulfite. When a yeast

culture was grown on dextrose media under similar conditions, the yeast continued to multiply and the yields of glycerol remained at the same level instead of dwindling to the vanishing point as they did in the case of the fermentation of maltose.

Several series were run using technical grade maltose as the fermentable substrate to see if the addition of nutrients, varying the strain of yeast, or altering the physical condition of the medium would increase the yields of glycerol. Results were inconclusive and often could not be rechecked. The highest yield again seemed to be in the neighborhood of 12 per cent for a four-day period of fermentation. Since technical-grade maltose contains 7 to 8 per cent dextrins, it was thought that the use of C. P. maltose which contains no dextrins would at least remove one complicating factor from consideration. The following experiments were carried out using the purified maltose, many of the experiments being similar to previous ones in which technical-grade maltose had been used. Steep water was used as the nutrient in most of the fermentations.

1. The effect of added materials on the fermentation of maltose in the presence of sodium sulfite

Since C. P. maltose is expensive and since the volume of medium is not as important as the surface-volume ratio, most of the maltose fermentations were carried out in 125-ml.

Erlenmeyer flasks containing 100 ml. of medium. Five per cent sugar solutions were also used in order to conserve the amount of maltose consumed. Yeast No. 53 was used for inoculating purposes, being carried on a 5-per cent maltose-steep water medium.

a. Preliminary experiments on the fermentation of maltose in the presence of 3 per cent sodium sulfite at an initial pH of 8.0 showed that the fermentation was not aided by the addition of malt, mold-bran, yeast extract, alfalfa, steep water, phosphates, or ammonium salts. The addition of dextrose caused fermentation to take place, but from the following data it appears that only the dextrose fermented. Mixtures of 3 drops of steep water, 5 gms. of sugars, and 90 ml. of water were sterilized in separate flasks. After the flasks had cooled, 10 ml. of inoculum and 3 gms. of sodium sulfite were added to each flask. All fermentations were run in duplicate, and the results of the analyses and the proportions of sugar used are shown in Table XXXIII.

It is readily apparent that the addition of dextrose did not aid the fermentation of maltose. Perhaps the dextrose might even be detrimental since, although the media were below pH 7.0, little maltose was fermented. The yeast evidently prefers dextrose to maltose under these conditions of fermentation.

Table XXXIII

Effect of Added Dextrose on the Fermentation of Maltose

Gms. Maltose	Gms. Dextrose	Gms. Glycerol	% Glycerol on dextrose*	% Glycerol on maltose**
4.0	1.0	0.21	21.0	0.1
2.5	2.5	0.59	23.6	3.0
0.0	5.0	1.03	20.6	---
5.0	0.0	0.03	---	0.6

*Calculated on the assumption that all glycerol came from dextrose.

**Calculated on the basis that 20.6 per cent of the dextrose was converted to glycerol; the remainder of the glycerol coming from the maltose.

b. It was noted that in several fermentations wherein the maltose appeared to ferment the initial pH was in the neighborhood of 7.0. The following series was prepared to see if the addition of nutrients would aid the fermentation of maltose when the initial pH was adjusted to 7.0. A solution of 80 gms. of maltose, 48 gms. of sodium sulfite, and 160 ml. of inoculum was made up to a total volume of 1600 ml. The medium was then adjusted to a pH of 7.0 and was distributed equally between sixteen flasks, to which had previously been added the various substances listed in Table XXXIV. The average analyses of the duplicate media are shown in the table. The fermentation period was four days.

Table XXXIV

Effect of Added Nutrients on the Fermentation of Maltose.

Material added	% Glycerol on maltose
0.1 gm. Malt	5.5
0.1 gm. Mold-bran	8.5
0.1 gm. Yeast extract	8.5
0.2 ml. Steep water*	10.7
0.1 gm. Dextrose	4.5
0.2 gm. $K_2HPO_4 \cdot 3H_2O$	3.1
0.2 gm. $K_2HPO_4 \cdot 3H_2O$ + 0.2 ml. steep water	11.2
Nothing added	5.3

*The steep water used in this and following experiments was sterilized since it was found that by sterilizing this substance contamination of the medium seldom occurred.

Steep water was evidently the best nutrient to be used, although yeast extract and mold-bran also aided the fermentation. The addition of phosphate in conjunction with steep water only gave an increase in yield of glycerol of 0.5 per cent, which was not sufficient to warrant its addition in further work.

2. The effect of pH on the fermentation of maltose in the presence of sodium sulfite

Since fairly good yields, as compared with the host of poor yields obtained in previous work with maltose, were found

when the initial pH had been adjusted to 7.0, several series were run to study the effect of the pH on the fermentation of maltose. The concentration of maltose in all of the fermentations in this series was 5 per cent, and the media were prepared in the same manner as those in the preceding experiment. The only differences were in the amounts of sulfite, and that steep water was used as a nutrient in all cases. After preparation the medium was divided into separate 200-ml. portions, the pH of each portion was adjusted to the desired level, and then each 200-ml. portion was divided equally between two 125-ml. Erlenmeyer flasks. After four days of fermentation the flasks were analyzed and the averaged results are shown in Table XXXV. From these data it is apparent that the optimum range of pH lies between 6.9 and 7.1 for the fermentation of maltose in the presence of sodium sulfite. The variation of the sulfite concentration does not seem to effect the optimum pH range or the maximum amount of glycerol obtainable. At pH values of 6.8 and below, it was noticed that the amount of total sulfite in the medium fell below the initial values and this was attributed to the formation of sulfurous acid and subsequent loss of sulfur dioxide by volatilization. Sulfurous acid is toxic to yeast so the decreasing glycerol yields with decreasing pH values are understandable. The lower yields of glycerol obtained above pH 7.1 can probably be attributed to the loss in activity of some enzyme system.

Table XXXV

Effect of pH on the Fermentation of Maltose in the Presence of Sodium Sulfite.

% Sulfite	Initial pH	Final pH	% Glycerol on maltose
3	6.4	-	3.5
3	6.8	-	10.1
3	7.2	-	11.6
3	7.6	-	4.3
3	8.0	-	0.8
4	6.8	6.7	11.1
4	6.9	6.9	11.4
4	7.0	7.0	11.6
4	7.1	7.1	12.2
4	7.2	7.2	9.8
5	6.8	6.0	8.7
5	6.9	6.8	11.6
5	7.0	7.0	11.5
5	7.1	7.1	11.3
5	7.2	7.2	9.9

Stark and Somogyi (1942) claimed that the optimum activity of maltase occurred at a pH of 6.0 to 6.8, but that the maximum rate of the fermentation of maltose occurred at pH 4.8. Since at pH 4.8 maltase was almost inactive, these investigators claimed that the fermentation of maltose was independent of maltase. Whether maltase is or is not necessary is still a controversial question. In any case, the pH range 6.9-7.1 is higher than either the pH range for the optimum fermentation of maltose or the pH of maximum activity of maltase. Previous experimental data in this thesis have indicated that sulfite became toxic to yeast when the pH fell below 6.8.

3. pH recordings of maltose fermenting in the presence of sodium sulfite

The yields of glycerol obtained from maltose heretofore amounted to about one-half of the yield that would be obtained from dextrose fermented under the same conditions. At first it was thought that perhaps only one-half of the maltose was fermented, but such a hypothesis did not seem reasonable. Another probable reason for the lower yields might simply be that the rate of fermentation of maltose was much less than that of dextrose. It had been noticed that maltose fermentations did not stop gassing after four or five days of fermentation, as did most dextrose fermentations.

Two similar media were fermented, one was allowed to ferment without pH control, the other was held at a pH level of 7.0-7.1. The media were prepared by dissolving 150 gms. of maltose, 120 gms. of sodium sulfite, and 6 ml. of sterile steep water in tap water and bringing the total volume to 2700 ml. The recorder electrode assembly was placed in the medium, and the pH adjusted to 7.0, after which adjustment 300 ml. of a 48-hr. culture of yeast No. 53 grown on a 5-per cent maltose-steep water medium was added and the pH brought to 7.1. Hydrochloric acid and sodium hydroxide were used to maintain the pH at the desired level in the pH controlled fermentation. Samples were removed at intervals and analyzed for fixed sulfite. The trend in the values for the fixed

sulfite parallels the amount of glycerol being formed in the fermentation. The results of these analyses are shown in Table XXXVI.

Table XXXVI

pH Recordings of Maltose Fermenting in the Presence of Sodium Sulfite.

Uncontrolled pH			Controlled pH		
Age in hrs.	pH	Ml. of 0.1 N I_2 for fixed sulfite	Age in hrs.	pH	Ml. of 0.1 N I_2 for fixed sulfite
5	7.1	0.3	17	7.1	0.7
23	7.0	0.8	41	6.9	2.6
46	6.8	2.8	65	6.9	4.6
70	6.7	4.1	90	7.0	5.4
94	6.7	5.3	113	7.1	6.5
118	6.7	6.1	137	7.1	6.9
142	6.7	7.0	161	7.0	7.6
166	6.7	7.8	185	7.0	8.1
190	6.7	8.5	209	7.0	8.4
214	6.8	9.0	233	7.0	8.9
238	6.9	9.8	281	7.0	9.5
286	7.0	10.5	329	7.0	9.8
310	7.3	10.9	377	7.0	9.9
338	7.3	11.2	-	-	-
362	7.3	11.4	-	-	-

Although in each fermentation the amount of fixed sulfite appeared to continue to increase, the rate of increase toward the end of the experiments had become so small that the titration error was even greater than the amount of increase. It was therefore concluded that the fermentations were finished for all practical purposes. In the case of the uncontrolled

fermentation the yield of glycerol amounted to 20.8 per cent of the initial maltose at the end of fifteen days of fermentation. For the controlled fermentation the yield was 18.5 per cent at the end of fifteen days. Although the controlled fermentation started to ferment at a more rapid rate than the uncontrolled fermentation, it gradually slowed up and then began to proceed more slowly than the latter. The final yields of glycerol obtained in these fermentations compared favorably with yields obtained from dextrose fermented under similar conditions, but the dextrose fermentations required a much shorter time.

An alcohol determination was made in duplicate on the fermented medium of the uncontrolled fermentation. A total of 35.5 gms. of alcohol was found, and of this amount 6.26 gms. originally came from the inoculum. When the total amounts of the products of the fermentation (glycerol, acetaldehyde, alcohol, and carbon dioxide) were calculated from the experimental data together with the theoretical equations for the alcohol and glycerol fermentations, only about 80 per cent of the maltose was accounted for. However, unfermented maltose may have been left in the medium. Other products such as acetic acid were not determined, and there was undoubtedly considerable loss of alcohol over the period of fifteen days.

V. SUMMARY AND CONCLUSIONS

1. The fermentation of enzyme-saccharified starchy materials in the presence of sulfites is not satisfactory. The cause of the poor fermentations may be attributed to the fact that the sugar formed from the starch by the enzymes is mostly maltose, and it has been found that the fermentation of maltose in the presence of sulfites does not proceed as well as does the fermentation of dextrose.

2. Solutions of acid-hydrolyzed starchy materials have proven suitable for the fermentative production of glycerol. The hydrolysis of dry-milled materials requires higher acid concentrations than does the hydrolysis of corn starch. The dry-milled materials would be cheaper, but the recovery of the glycerol from the fermented solutions might be more difficult than in the case of acid-hydrolyzed corn starch.

3. Dextrose, levulose, and sucrose can all be fermented satisfactorily in the presence of sulfites. Maltose, lactose, and trehalose do not ferment satisfactorily.

4. Maltose may be fermented in the presence of sulfites by furnishing suitable nutrients for the yeast and by adjusting the pH to 6.9-7.1, although the fermentation takes considerably longer than does the fermentation of dextrose.

5. Among those strains tested, the strain of yeast has been shown to have little effect on the yields of glycerol when dextrose, sucrose, or levulose is used as the fermentable substrate. Certain strains of yeast appear to be more satisfactory when maltose is the sugar fermented.

6. The surface-volume ratios of the fermentation media influence the yields of glycerol. When the ratios rise above about two-tenths, the yields of glycerol, determined by the iodine-titration method, begin to decrease.

7. The yields of glycerol are never as great when magnesium sulfite is used as the acetaldehyde-binding agent as when the more soluble sodium sulfite is employed in high concentrations in conjunction with large inocula. When ordinary inocula are used the yields of glycerol are about in the same range for either sulfite. It has been found that the maximum yields of glycerol obtained when magnesium sulfite is employed are in the range from 20 to 25 per cent of the initial dextrose. When sodium sulfite is used in 10-per cent concentration, a yield of about 30 per cent glycerol on initial dextrose is obtained in the fermentation of a 10-per cent dextrose solution by a large amount of yeast.

8. The amount of yeast present in a fermentation medium affects the yields of glycerol when sodium sulfite is used as the aldehyde-binding agent. Ordinary inocula do not furnish

enough yeast for the fermentation of dextrose when the sodium sulfite concentrations exceed 3 to 5 per cent, depending on the sugar concentrations. Above these sulfite concentrations the yeast does not multiply or, perhaps, multiplies very slowly. Ordinary inocula suffice when magnesium sulfite is used as the aldehyde-binding agent.

9. When the concentration of yeast is less than 50,000,000 cells per ml. of medium the yields of glycerol may be improved by the gradual addition of the sodium sulfite to the fermenting medium.

10. Yeast metabolism in a fermenting medium containing sulfite tends to adjust the reaction in such a way as to obtain the best possible conditions for growth and fermentation. It has been found that in fermenting media containing magnesium sulfite the pH decreases to 6.5-6.6 and remains there until the fermentation is nearly completed. When sodium sulfite is used, the pH decreases to about 7.0 and stays at that level until the fermentation is almost over.

11. In general it may be said that those factors which affect the velocity of an enzymatic reaction affect the fermentative production of glycerol. These factors include the concentration of substrate, the concentration of enzyme (yeast), the concentration of electrolyte, the temperature, the pH of the medium and the presence of activators and

inhibitors (sulfite). In the case of the glycerol fermentation the ratio of the sulfite in solution to the fermentable substrate is also an important factor.

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